Identification of transcripts associated with postharvest withering in *Vitis vinifera* L. 'Cabernet Franc' grape berry

Kimberley Cathline, B.Sc. (Hons, Distinct)

Biological Sciences

Submitted in partial fulfilment
of the requirements for the degree of

Master of Science

Faculty of Mathematics and Science, Brock University
St. Catharines, Ontario

Abstract

The withering of grape berries following harvest is known in Italian as *appassimento*, and it used as a tool to alter the composition of the berries prior to winemaking. Specific gene expression patterns were determined by RNA Sequencing in the red-skinned berries of *Vitis vinifera* L. 'Cabernet Franc' for three time points during withering, at both slow and fast rates, up to 40.7 and 31.2 percent cluster weight loss respectively, with a target of 29 $\pm$ 1 ^{o}Brix. Slow withering was associated with a higher number of differentially expressed genes, and those commonly up-regulated across all time points were associated with a higher number of gene ontology categories, including phenylpropanoid and cellular aromatic compound metabolism and biosynthesis. Commonly up-regulated genes across fast drying were only related to response to abiotic and biotic stimuli terms. Both slow and fast withering were associated with an up-regulation of numerous WRKY and MYB transcription factors, as well as stilbene synthase, phenylalanine ammonia lyase and trehalose-phosphate phosphatase. With respect to anthocyanin biosynthesis, there was no change in expression for *MYBA* genes, and UDP-glucose:flavonoid 3-O-glucosyltransferase was down-regulated transiently in fast withering and showed no change in expression in slow. Slow withering was also associated with an up-regulation of a dehydrin and the *CBF3* gene. The results of the present study suggest that fast withering may be associated with primarily an intense stress response, while slow withering may be associated with a senescence or over-ripening, in combination with a gradual adaptation to a less intense dehydration stress. As such, the influence of rate during withering will likely have a strong impact on the final berry composition, and thus will influence the characteristics of the resulting wines.

Acknowledgements

I would like to thank Dr. Vincenzo De Luca for the opportunity to be a member of his laboratory and to carry out my MSc thesis work under his supervision. I am grateful for his mentorship, patience and guidance through this process. I would also like to thank my co-supervisor Dr. Bernard Goyette, who paved the way for me to pursue the opportunity of graduate studies. His constant support of this endeavour will always be remembered. In addition, I would like to thank my supervisory committee members, Dr. Debbie Inglis and Dr. Charles Després, for their guidance and input over the course of my research.

This research would not have been possible without the financial support of Vineland Research and Innovation Centre, with partial funding provided through Growing Forward 2, as well as funding provided by the Cool Climate and Oenology and Viticulture Institute, through the Ontario Research Fund – Research Excellence. I would also like to thank Graham Rennie (Rennie Estate Winery) for kindly providing the grapes necessary for the project.

The assistance, advice and support I have received from my peers and colleagues has been indispensable throughout this research. I would like to give special thanks to Dr. Paulo Cázares for his mentorship, and for his ideas and insight. I would like to thank Dr. Kyung-Hee Kim for acquainting me with Brock and the De Luca lab, and for teaching me the fundamentals of RNA extraction technique. I would like to thank Travis Banks for his assistance in the bioinformatics portion of this research, and for his insight and suggestions. I would like to also thank Dr. Tania Humphrey, Dr. Gideon Avigad, Dave Vuyk, Joseph O'Neill, Dr. Amy Bowen, Dr. Isabelle Lesschaeve, Dr. Matthew Fidelibus, Dr. Jim Brandle, Katie Woolfson, Dr. Vince Qu and Dr. Brent Wiens.

Most importantly, I would like to thank my husband, Giovanni Rodríguez M., for his unending encouragement, support and optimism. He has been an anchor for me throughout this journey, and I would have been lost without him speaking into my life each day. I would also like to thank my daughter Scarlette for her patience, for reminding me what is important, for her joyful outlook on life and for helping me to see the world through her eyes. I would like to thank my parents, Brian and Marliene Cathline, and my sisters Amanda and Christina for their uplifting words, support and understanding. I would like to also thank my mother-in-law Veronica for keeping our home running during these past few months of studies. Finally, I would like to thank my God, for being with me every step of the way, for helping me, for giving me peace, for His guidance, wisdom and friendship.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

4CL – 4-coumarate-CoA ligase

ABA – Abscisic acid

ACC oxidase – 1-aminocyclopropane-1-carboxylate oxidase

AFLP-TP – Amplified fragment length polymorphism-transcriptional profiling

ADH – Alcohol dehydrogenase

ANOVA – Analysis of variance

AP2/ERF – APETALA2 ethylene response factor

Borkh. – Moritz Balthasar Borkhausen

bp – Base pairs

CBF – C-repeat binding factor

cDNA – Complimentary deoxyribonucleic acid

CH4 – Cinnamate 4-hydroxylase

CHS – Chalcone synthase

COR – Cold regulated

CRT – C-repeat

DE – Differentially expressed

DNA – Deoxyribonucleic acid

DRE – Dehydration response element

DREB – Dehydration response element binding factor

E-L – Eichhorn-Lorenz

ERF – Ethylene response factor

FDR – False discovery rate

FLS – Flavonol synthase

FW – Fast withering

GO – Gene ontology

H – Harvest

Heynh. – Gustav Heynhold

L. – Carl Linneaus

L.H. Bailey – Liberty Hyde Bailey

LOX – Lipoxygenase

Mig. – Emil Friedrich August Walter Migula

Michx. – André Michaux

mRNA – Messenger RNA

NCBI – National Centre for Biotechnology Institute

NGS – Next-generation sequencing

ORF – Open reading frame

PAL – Phenylalanine ammonia lyase

PC1 – Principal component 1

PC2 – Principal component 2

PEAR – Paired-end read merger

Pers. – Christiaan Hendrik Persoon

qPCR – Quantitative polymerase chain reaction

RH – Relative humidity

RNA – Ribonucleic acid

RNA-Seq – RNA Sequencing

rRNA – Ribosomal RNA

ROS – Reactive oxygen species

RPC – Reusable plastic container

RT-PCR – Reverse transcription polymerase chain reaction

STAR – Spliced transcripts alignment to a reference

STS – Stilbene synthase

SW – Slow withering

S. Watson – Sereno Watson

TF – Transcription factor

Torr. – John Torrey

TSS – Total soluble solids

UFGT – UDP-glucose:flavonoid-3-O-glucosyltransferase

Chapter 1. Introduction and Literature Review

1.1 Appassimento wine

Wine produced from grapes which have undergone postharvest withering is referred to in Italian as *appassimento*. *Appassimento*-style wine production is based on a traditional technique of drying grapes to produce a rich premium wine or a fine sweet wine, which are commonly known as Amarone or Recioto respectively. Amarone wine is a high-quality, distinctive red wine with special sensory attributes, which is produced in the region of Valpolicella in north-eastern Italy, whereby slow drying traditionally occurs in barns under natural environmental conditions (Paronetto and Dellaglio, 2011). The withering process can last from 90 to 150 days (Accordini, 2013) and grapes are usually crushed when they have lost 35 to 40% of their original weight (Paronetto and Dellaglio, 2011; Tonutti *et al*., 2004). This moisture loss produces changes in the composition and the chemical-physical properties of the berries, which concentrates and develops aromatic compounds, sugars and polyphenols, and in turn affects the flavour and aroma of the resulting wine (Bellincontro *et al*., 2004; Bonghi *et al*., 2012; Paronetto and Dellaglio, 2011). Several cultivars are used in the production of Amarone, and these are required to include 'Corvina' and 'Rondinella' in specific proportions (Paronetto and Dellaglio, 2011).

Beyond Italy, the *appassimento* technique is also a wine-making tool of interest in cool climate regions such as Ontario, Canada, which experiences a relatively short growing season, especially in terms of red grape production. The climate in Ontario in the months after harvest make drying grapes in uncontrolled environments quite challenging, due to berry damage and loss from pests, animals and weather, as well as the development of fungal infection and disease. In recent years, various drying technologies have been

developed to assist with controlling postharvest withering through modulation of the environmental parameters, which in turn affects the rate of dehydration (Tonutti *et al*., 2004). Controlled withering creates the ability to maintain high quality grapes over a postharvest period of 100 days or more in regions such as Ontario, where it would otherwise be extremely difficult, and in Italy it reduces the risk associated with dependence solely on the climate. The use of drying technologies in *appassimento*-style wine production has also allowed for the ability to reduce the traditional 100-day withering period and to dry grapes rapidly over a matter of weeks.

Postharvest withering for *appassimento*-style wine production induces changes in the berry which are the result of water loss and concentration of metabolites, but are also due to the modulation of metabolism in response to dehydration stress (Pedreschi and Lurie, 2015; Tonutti and Bonghi, 2013). Typical characteristics of partially withered berries include increased sugar concentration, reduced titratable acidity, modified volatile and phenolic profiles, as well as an increased tartaric-to-malic acid ratio and decreased glucose-to-fructose ratio (Accordini, 2013; Bellincontro *et al*., 2004; Chkaiban *et al*., 2007; Rizzini *et al*., 2010; Tonutti and Bonghi, 2013).

1.2 Postharvest withering of grape berries

1.2.1 Ripening and senescence in grape

The process of senescence is the final phase of fruit ontogeny and consists of the initiation of a series of normally irreversible events beginning at the inception of ripening, which eventually lead to cellular breakdown and death (Sacher, 1973). Ripening is recognized as a senescent phase, and in fleshy fruit, ripening senescence is the dominant phase of ontogeny (Blackman and Parija, 1928). The development of the common grape

berry, *Vitis vinifera* L., follows a double sigmoid pattern of growth (**Figure 1.1**), with ripening initiated at the beginning of the second development phase (Coombe, 1995). The onset of ripening in grape is known as *véraison*, which is characterized by a reduction in titratable acidity, an increase in sugar accumulation, softening and enlargement of berries, accumulation of flavour compounds and the initiation of anthocyanin production (Chervin *et al*., 2004, Coombe, 1995; Robinson and Davies, 2000).

Ripening in fruit is viewed as a dynamic process involving structural, biochemical and physiological changes, which eventually lead to the production of an edible fruit and then continue beyond this point to end in cell death (Tonutti and Bonghi, 2013). There is an abundance of information available on the physiological, molecular and biochemical changes associated with grape berry ripening up to the point of optimal edible composition. However, less biochemical and molecular information exists regarding those phases of over-ripening and senescence (Tonutti and Bonghi, 2013). These phases in grapes can be studied both on-the-vine, or investigated in clusters following harvest.

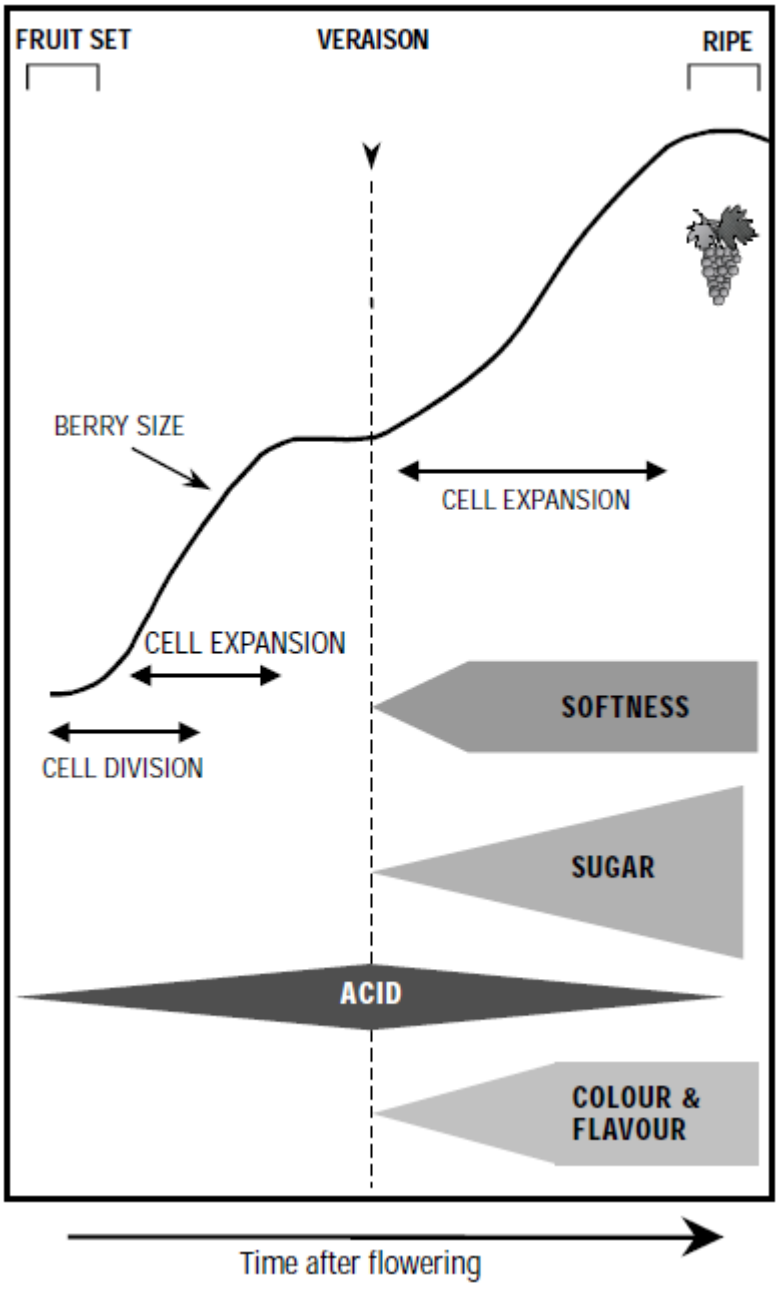


Figure 1.1 Schematic representation of grape berry development. The curve indicates changes in berry size. The inception of ripening (*véraison*) is shown by the vertical dashed line. Softening, sugar accumulation, metabolism of organic acids and synthesis of colour and flavour compounds all occur after *véraison* (Robinson and Davies, 2000).

After crops are severed from the plant at harvest they are still alive and respiring, and grapes, like all perishable crops, continue to metabolize actively until cell death occurs, reacting to environmental stimuli and stresses, which in turn affects their composition (Grierson, 2002; Pedreschi and Lurie, 2015; Rizzini et al., 2009; Tonutti and Bonghi, 2013). The metabolism of detached grapes will be different from the metabolism of berries on-the-vine, which is an important factor in *appassimento* withering. This difference in metabolism is due to both the lack of attachment to a supply of water, minerals and energy from the vine's vasculature, and also as a result of the specific environmental conditions and stresses to which the berries are exposed during the postharvest period (Pedreschi and Lurie, 2015; Tonutti and Bonghi, 2013).

1.2.2 Postharvest dehydration stress

Once grape clusters are detached from the mother plant they can be exposed to a range of stresses including dehydration (Pedreschi and Lurie, 2015). Postharvest water stress is greater than water stress experienced on-plant, as the harvested crop is dependent solely on its internal water supply, and cells cannot overcome this stress through vascular uptake (Cirilli *et al*., 2012; Pedreschi and Lurie, 2015; Zamboni *et al*., 2008). Postharvest grapes are also affected by the rate and intensity of water loss, which increases ethylene production and respiration, induces cell wall enzyme activity, and impacts polyphenol and volatile levels (Bellincontro *et al*., 2004; Costantini *et al*., 2006). The extent and rate of dehydration in the grapes during withering is influenced by the specific postharvest conditions (Chkaiban *et al*., 2007).

As water stress increases in perishable fruits, changes in metabolism and respiration occur, as well as loss of cellular turgor pressure and accumulation of solutes (Hsaio, 1973).

In order to cope with abiotic stresses such as drought, plants and their harvested products react by the induction or repression of specific stress-responsive genes, which in turn affect metabolic processes (Pedreschi and Lurie, 2015; Xiao *et al*., 2006). Abiotic stress in plants generates a number of common responses including the accumulation of "compatible" metabolites such as trehalose, sorbitol, mannitol and the amino acid proline, which protect them against water loss. In addition, the biosynthesis of secondary metabolites (polyphenols) that function as stress protectants occurs and scavenging enzymes for reactive oxygen species (ROS) are accumulated (Pedreschi and Lurie, 2015). Furthermore, drought, heat and cold stress in plants also share similar response features, in that they all have a common dehydration element. The dehydration response specifically includes: accumulation of compatible osmoprotectants such as proline, sugars and sugar-alcohols; changes in carbon metabolism and glycolysis; accumulation of anthocyanin and phenylpropanoid metabolites; induction of C-repeat binding factor (CBF) transcription factors which are involved in regulation of cold acclimation gene expression; and accumulation of ROS scavenging enzymes (Pedreschi and Lurie, 2015).

Postharvest water stress is often exploited in grapes in order to alter their physical or biochemical structure for the purposes of generating completely dehydrated berries (raisin production), or to create partially withered berries used in the production of various wines, such as the previously described *appassimento*-style wines (Bellincontro *et al*., 2004; Rizzini *et al*., 2009). There is limited information with respect to the effect of water stress on the metabolism, biochemistry and physiology of grape berries after harvest (Rizzini *et al*., 2009), and very little is known in any fruit about the effects of the postharvest stage and associated stresses specifically at the molecular level (Zamboni *et*

al., 2008). Postharvest studies in withering grapes have shown that there is an active modulation of metabolic pathways, including stress-related responses and secondary metabolism, and have also demonstrated that berries are still metabolically and transcriptionally active at advanced stages of dehydration (Di Carli *et al*., 2011; Versari *et al*., 2001).

1.2.3 The grape berry withering response and associated factors

The response of grapes to postharvest withering is determined by both exogenous and endogenous factors; that is, the drying conditions, as well as the physical and biochemical properties of the berries themselves (Mencarelli and Bellincontro, 2013). The main environmental conditions which are considered during drying are the air flow rate, the temperature and the relative humidity (RH), which have been shown to affect berry metabolism (Bellincontro *et al*., 2004; Mencarelli and Bellincontro, 2013). The behaviour of different grape varieties during drying depends on the berry's specific characteristics, including their surface to volume ratio, skin thickness, micropore abundance, presence of cracks and cuticle thickness (Mencarelli and Bellincontro, 2013).

An initial stress response during postharvest withering was associated with a shift from aerobic to anaerobic metabolism when grapes sustained a 10 to 15% cluster weight loss (Bellincontro *et al*., 2004). This initial response was confirmed by Costantini *et al*. (2006), who additionally observed increases in lipoxygenase (LOX) activity, as well as in concentrations of abscisic acid (ABA) and proline. ABA is a plant hormone involved in numerous processes including modulation of water stress (Ferrandino and Lovisolo, 2014), and proline can function as a osmoprotectant during dehydration (Penna *et al*., 2006). LOX is an oxidative enzyme involved in cell protection, and during dehydration its activation

may be due to the presence of ABA and/or the modification of cell structure as a result of turgor loss (Costantini *et al*., 2006). Costantini *et al*. (2006) also observed a second metabolic stress response at greater than 19.5% weight loss, which was associated with an increase in total protein and proline content. In addition, the researchers observed an increase in alcohol dehydrogenase (ADH) activity and ethyl acetate content. The first 5 to 10% cluster weight loss does not apparently affect the main metabolism of the berry, as it is primarily due to water loss from the rachis (stems and cluster branches), which creates a pressure differential and draws a limited amount of water from the berry, a process which continues until the rachis is completely dry (Centioni *et al*., 2014; Cirilli *et al*., 2012; Mencarelli and Bellincontro, 2013). The rachis is highly permeable due to the presence of numerous stomata and lenticels, while the berry does not readily lose moisture, as it has no stomata and is covered in a thick waxy cuticle layer. Once the rachis is dry, cluster water loss is due solely to transpiration from the berries which begin to experience dehydration stress in response to the environmental conditions (Cirilli *et al*., 2012).

The rate and intensity of the dehydration process both play an important role in the postharvest stress response of perishables (Kays, 1997), as well as the resulting properties of the grape berries, with consequences to the quality of the wines (Tonutti *et al*., 2004). Since grapes are alive following harvest, their metabolism during withering will impact the compounds they produce, which in turn contributes to the wine quality. As a result, if the drying process is too fast, there is no opportunity for further development of compounds and aromas (Mencarelli and Bellincontro, 2013). Such is the case for raisins, where the goal is only a concentration of sugars, and achieving an aromatic product is not a concern, thus a very rapid and high temperature drying process is employed. However, if the goal is

to produce a grape with a composition that is suitable for the production of a high-quality wine, it is imperative to carefully manage the rate and intensity of the drying parameters, as both have an effect on the withering process and subsequently impact the metabolism and composition of the berry (Barbanti *et al*., 2008; Mencarelli and Bellincontro, 2013; Rolle *et al*., 2013).

Temperature is a key factor modulating both the speed of grape dehydration as well as their metabolism, with higher respiration rates observed in berries withered at 20 ^{o}C as compared to 10 ^{o}C (Bellincontro *et al*., 2009). The rate of dehydration affected the range of volatiles produced by the berries (Bellincontro *et al*., 2004), as well as the total phenol and anthocyanin content (Bellincontro *et al*., 2004; Bellincontro *et al*., 2009). Lower temperatures (10 ^{o}C or under) required more drying time while generating less oxidation, maintaining varietal volatiles (Cirilli *et al*., 2012; Mencarelli and Bellincontro, 2013) and resulted in improved berry structure during the withering process (Bellincontro *et al*., 2009). Maintenance of the cellular structure allows for water to move slowly from cell to cell, decreasing water stress and allowing for a slower production of important secondary metabolites such as volatiles and polyphenols, along with slower anaerobic fermentation, which delays the production of metabolites such as ethanol, acetaldehyde and acetic acid (Crilli *et al*., 2012; Mencarelli and Bellincontro, 2013).

Air speed also affects berries during dehydration, with higher ventilation (2.5 m/sec) at 10 ^{o}C associated with an increase in respiration and drying rate, as compared to lower ventilation (1.5 m/sec) at the same temperature (Bellincontro *et al*., 2009). In addition, RH is an important factor to consider in the drying process. As expected,

decreasing the RH during postharvest withering of grapes has been demonstrated by Barbanti *et al*. (2008) to increase the rate of dehydration.

1.3 Molecular changes during postharvest withering

Only a small number of studies have investigated the response of grape berries to postharvest withering at the gene expression level. Changes in metabolism during postharvest withering in grape were initially demonstrated to be controlled at the gene level by Versari *et al*. (2001). Stilbene synthase (STS) catalyzes the biosynthesis of resveratrol (*trans*-3,5,4'-trihydroxystilbene) in grape, and Versari *et al*. (2001) investigated the accumulation of STS messenger RNA (mRNA) in 'Corvina' berries. The researchers employed a Northern blot analysis to demonstrate an up-regulation of the *STS* gene, accompanied by an accumulation of resveratrol during the dehydration process. A similar observation was made by Cirilli *et al*. (2012), who demonstrated that sensitivity to dehydration stress occurred at the gene level for ADH, before evidence of other physiological effects became apparent. The researchers investigated ADH activity over time, along with its gene expression through the use of quantitative polymerase chain reaction (qPCR) analysis. They demonstrated an up-regulation of the *VvAdh2* gene prior to a corresponding increase in ADH activity.

The use of transcriptome analysis allows for the study of the expression behaviour and transcript accumulation of a large number of genes, and over the past decade, five separate transcriptome analysis studies have been reported during postharvest withering of grape berries. These analyses have employed both a microarray (Fasoli *et al*., 2012; Rizzini *et al*., 2009; Zamboni *et al*., 2010; Zoccatelli *et al*., 2013) and amplified fragment length polymorphism-transcriptional profiling (AFLP-TP) approach (Zamboni *et al*., 2008). The

studies were conducted using ‘Corvina’, ‘Sangiovese’ and ‘Oseleta’ berries (Zoccatelli *et al*., 2013) and de-seeded ‘Corvina’ grape berries which were withered in uncontrolled natural environmental conditions (Fasoli *et al*., 2012; Zamboni *et al*., 2010; Zamboni *et al*., 2008), as well as using the skins of ‘Raboso Piave’ berries in a comparison of both controlled fast withering and uncontrolled slow natural withering conditions (Rizzini *et al*., 2009).

Transcriptome analysis of berries during postharvest dehydration has revealed a unique withering response, involving the modulation of specific genes during the process that are not modulated during on-plant drying (Zamboni *et al*., 2008). In addition, the rate and intensity of postharvest withering has been shown to affect the distribution of expressed genes within functional categories. Furthermore, an increased intensity of water loss from 10 to 30% cluster weight has demonstrated a greater number of differentially expressed probes, indicating that grape berries are still transcriptionally active, even at high levels of dehydration (Rizzini *et al*., 2009). Common genes characterizing postharvest withering in grapes include those involved in abiotic and biotic stress responses, transport, carbohydrate and secondary (phenolic) metabolism, regulation of transcription (transcription factors) and ethylene metabolism (Bonghi *et al*., 2012; Cirilli *et al*., 2012; Mencarelli *et al*., 2010; Rizzini *et al*., 2009; Tonutti *et al*., 2004; Tonutti and Bonghi, 2013; Versari *et al*., 2001; Zamboni *et al*., 2008).

1.4 Functional and regulatory withering stress responses

Plants react to abiotic stresses such as drought and cold temperature by the induction or repression of specific stress-responsive genes, which in turn affect various developmental, physiological and biochemical processes (Xiao *et al*., 2006). Stress-

responsive genes produce proteins which can have a functional role, such as antifreeze and water channel proteins, or those that alter the levels of various metabolites such as proline, sugars and sugar alcohols. Stress-responsive genes can also produce proteins with a regulatory role (Akhtar *et al*., 2012), which includes a network of transcription factors (TFs) that control a cascade of genes involved with stress modulation in plants (Mizoi *et al*., 2012). Transcription factors are able to bind to specific elements in promoter regions of target genes and in doing so they regulate their expression.

1.4.1 CBF transcription factors

Although grape has been considered a non-climacteric fruit, meaning that ripening is independent of the plant hormone ethylene, it has been demonstrated in recent years that a weak increase in the production of ethylene is experienced by berries at the onset of ripening (Chervin *et al*., 2004). This peak in ethylene production is coupled with an increase in the activity and transcript accumulation of the enzyme responsible for the last step of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate (ACC) oxidase (Chervin *et al*., 2004). In addition to its role in ripening, ethylene is associated with stress responses, and an increase in the amount of mRNA from genes encoding ethylene biosynthesis and response functions was seen in the berries of vines subjected to water-deficit conditions, including ACC oxidase (Grimplet *et al*., 2007). An up-regulation of ACC oxidase, independent of the rate and intensity of dehydration (Rizzini *et al*., 2009), and also an up-regulation of *S*-adenosyl methionine synthase, which is involved in ethylene biosynthesis (Zamboni *et al*., 2008), has been shown during withering in grape berries, indicating that ethylene may be involved in the postharvest water stress response.

The role of ethylene in response to stress is likely regulated by transcription factors, such as those in the APETALA2 ethylene response factor (AP2/ERF) family, which share a conserved DNA-binding domain (Yin *et al*., 2012; Zhuang *et al*., 2009). There are five subfamilies of AP2/ERF, including the dehydration response element binding factor (DREB) subfamily and the ethylene response factor (ERF) subfamily. *ERF* genes have only been studied in a few species of plants and almost all reported stress-related investigations have been performed on vegetative tissues. A recent study was conducted using harvested kiwifruit that were subjected to low and high temperature, high CO_2 and high water loss conditions, and it was found that *ERF* genes were important for modulating the postharvest stress response (Yin *et al*., 2012). C-repeat binding factor/DREB1 (CBF/DREB1) proteins are members of the DREB subfamily and are collectively referred to as CBF proteins; these transcription factors bind to C-repeat (CRT) or dehydration-responsive elements (DRE) to activate cold regulated (*COR*) genes in the CBF pathway, whose expression convey tolerance to low temperatures and drought (Siddiqua and Nassuth, 2011; Xiao *et al*., 2006). A CBF encoding gene was originally discovered in *Arabidopsis thaliana* (L.) Heynh., with a total of six now identified in this plant; CBF proteins have since been found in a large number of different plants (Akhtar *et al*., 2012; Siddiqua and Nassuth, 2011).

Four *CBF* genes, *CBF 1-4* have been identified in the temperature-sensitive *Vitis vinifera*, as well as in the wild, freeze-tolerant *Vitis riparia* Michx. (Xiao *et al*., 2006; Xiao *et al*., 2008). Transcripts of *VvCBF1*, *2* and *3* were shown to accumulate to relatively high levels in detached young *V. vinifera* 'Chardonnay' leaves under drought stress within 15 minutes after exposure, and these genes were also induced after exposure to low

temperature (4 ^{o}C) treatment in both *V. vinifera* and *V. riparia* young leaves (Xiao *et al*., 2006). Although no *CBF* genes have been reported thus far to be modulated in grape berries during postharvest withering, the up-regulation of two ethylene biosynthesis genes during drying, along with the accumulation of *CBF* gene transcripts in response to drought in grape leaves suggests that this could be an area worthy of investigation.

1.4.2 WRKY transcription factors

Members of the WRKY transcription factor family are part of a large group of regulatory proteins which show high binding affinity to the W-box DNA promotor sequence, and are primarily involved in biotic and abiotic stress tolerance in plants (Pandey and Somssich, 2009; Ülker and Somssich, 2004). These transcription factors all share the WRKY DNA-binding domain, comprising a superfamily with 74 members in *Arabidopsis* and 59 in *V. vinifera* (Wang *et al*., 2015).

Postharvest withering in grape berries has induced the up-regulation of a transcript which shows homology to the WRKY6 transcription factor from *Nicotiana attenuata* Torr. ex S.Watson (Zamboni *et al*., 2008). In numerous plant species, *WRKY* genes show rapid and strong up-regulation in response to wounding, drought, hot and cold temperature, as well as to biotic stress (Ülker and Somssich, 2004). The accumulation of transcripts for a putative WRKY transcription factor during appassimento drying indicates that these proteins may have a role in the postharvest withering stress response in grape berry (Zamboni *et al*., 2008).

1.4.3 MYB transcription factors

MYB transcription factors share the MYB binding domain and have been found to accumulate in plants in response to environmental stimuli, such as light and salt stress

(Martin and Paz-Ares, 1997). There are estimated to be over 100 MYB transcription factors in *Arabidopsis* and some of these proteins are also known to be involved in the control of phenylpropanoid metabolism (Martin and Paz-Ares, 1997).

Many phenylpropanoid compounds play a role in abiotic and biotic stress tolerance, such as the flavonoids and stilbenes. In both tobacco and *Antirrhinum majus* L., commonly known as snapdragon, the MYB305 protein activated the gene for phenylalanine ammonia lyase (PAL), which encodes for the enzyme responsible for the first step of the phenylpropanoid biosynthetic pathway (Martin and Paz-Ares, 1997). Transcriptome profiling of grape berries during postharvest withering identified homologues to apple MYBR2 (Zamboni *et al*., 2008) and to *Vitis labruscana* L.H. Bailey MYBB (Bonghi *et al*., 2012) transcription factors, which were both up-regulated and might be linked to changes in phenylpropanoid metabolism during drying. In contrast, the down-regulation of a *MybA* transcription factor during postharvest withering has also been observed (Bonghi *et al*., 2012; Rizzini *et al*., 2009). MYBA is responsible for the control of anthocyanin biosynthesis and its repression during drying is opposite to what has been observed in berries from vines that have experienced water-deficit conditions (Castellarin *et al*., 2007; Grimplet *et al*., 2007), thus suggesting that anthocyanin biosynthesis is affected in different ways by each process.

1.4.4 Phenylpropanoid metabolism

Phenylpropanoid metabolism is a type of secondary metabolism in plants involving changes to compounds which are initially derived from phenylalanine, including simple phenols, stilbenes and flavonoids (**Figure 1.2**). Anthocyanins and flavonols belong to the group of phenolic compounds called the flavonoids. The concentration and ratios of

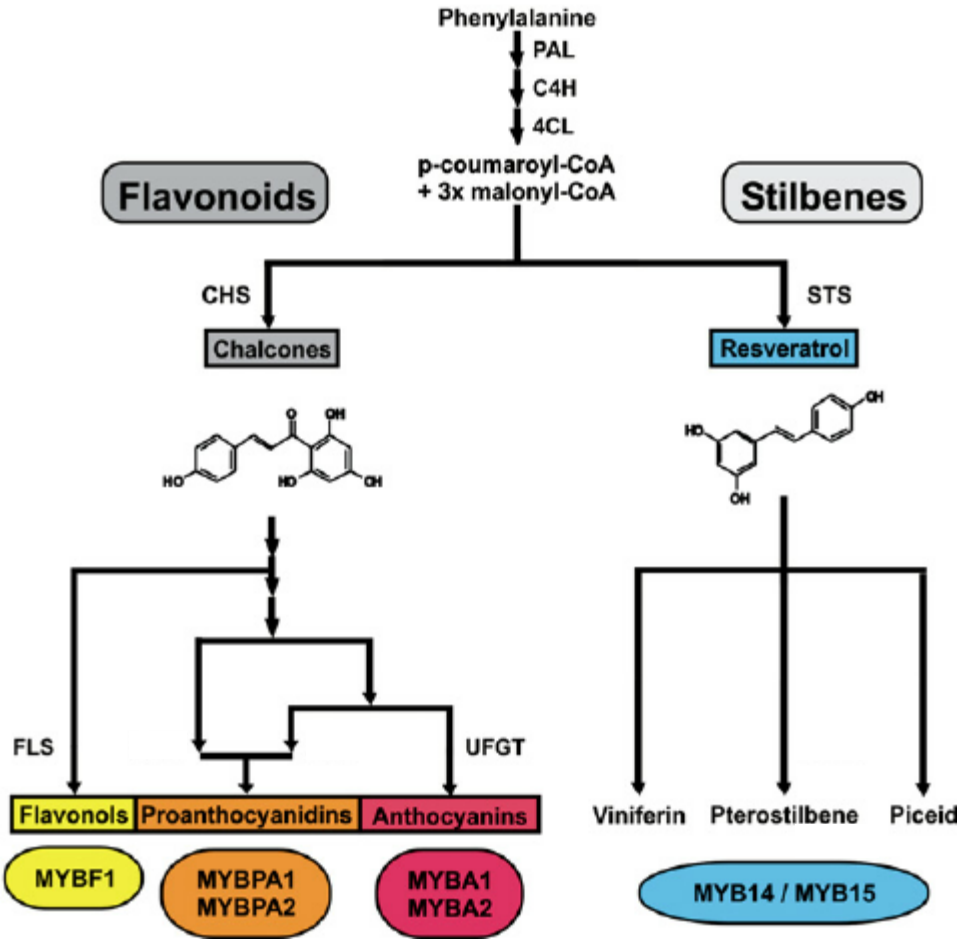


Figure 1.2 Simplified representation of the grapevine phenylpropanoid biosynthetic pathway leading to the production of flavonoids and stilbenes. Transcriptional regulation of the general enzymes is conducted by MYB transcription factors including MYBF1, MYBPA1, MYBPA2, MYBA1, MYBA2, MYB14 and MYB15. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; FLS, flavonol synthase; UFGT, UDP-glucose:flavonoid-3-O-glucosyltransferase; STS, stilbene synthase (Höll *et al*., 2013).

different phenolic compounds are important in determining the quality of wine, with the colour of red wines due to the presence of anthocyanin pigments, and astringent and bitter properties mainly a result of flavan-3-ols and flavonols (Corradini and Nicoletti, 2013; Moreno *et al*., 2008). Beyond their role in wine quality, phenolics are also involved in plant defense. Anthocyanins and flavonols have antioxidant capabilities, and it is suggested that they may also be able to absorb ultraviolet light, in addition to various other protective roles (Chalker-Scott, 1999; Martin and Paz-Ares, 1997). Numerous flavonoids, along with other phenylpropanoids such as stilbenes, are involved in abiotic and biotic stress responses (Martin and Paz-Ares, 1997).

The literature contains conflicting views regarding changes in concentrations of various phenolic compounds during postharvest withering, which is likely due to the variability in drying conditions, rate and intensity of dehydration, as well as type of tissue and grape variety used (Tonutti and Bonghi, 2013). With respect to molecular changes in phenylpropanoid metabolism during drying, an increase in transcripts coding for phenylalanine ammonia lyase (PAL) has been shown in both fast and slow drying withering treatments (Bonghi *et al*., 2012; Rizzini *et al*., 2009; Tonutti *et al*., 2004; Zamboni *et al*., 2008). PAL represents the first step in the phenylpropanoid pathway (**Figure 1.2**) and this protein is encoded by a multigene family. In addition to an up-regulation of some *PAL* gene members during withering, Bonghi *et al*. (2012) also observed a selective down-regulation of two other *PAL* genes across different drying conditions. The increase in transcripts of *PAL* observed during postharvest drying (Bonghi *et al*., 2012; Rizzini *et al*., 2009; Tonutti *et al*., 2004; Zamboni *et al*., 2008) is similar to what has been observed in the field following water-deficit (Grimplet *et al*., 2007). The rate of drying has a notable effect on

the expression pattern of *PAL*, which was demonstrated by Tonutti *et al*. (2004) in 'Corvina' grape berries, where an increase in transcripts in fast dry samples was only detected at the end of the withering period at 42% cluster water loss, while slow dry samples showed accumulation beginning at 14% water loss.

The postharvest withering process appears to induce a general activation of the phenylpropanoid pathway due to an increase in *PAL* transcripts. However, gene expression for pathways involved in the biosynthesis of various classes of phenylpropanoids appears to be affected differently. Using Northern Blot analysis, transcripts encoding for chalcone synthase (CHS), the first committed step in flavonoid biosynthesis, were reported to remain at levels similar to harvest in 'Corvina' berries throughout withering in both fast and slow drying conditions (Tonutti *et al*., 2004). However, two *CHS* genes were down-regulated in skins of 'Raboso Piave' berries during both slow and rapid rates of postharvest withering at 30% cluster weight loss (Bonghi *et al*., 2012). The down-regulation of *CHS* genes suggests a general suppression of the flavonoid pathway, however one category of flavonoids, the flavonols, appears to be positively affected by the withering process (Tonutti and Bonghi, 2013). In skins of 'Raboso Piave' berries dehydrated up to 30% cluster weight loss, flavonol synthase (*FLS*) was induced, coupled with an increase in the concentration of the flavonol quercetin (Bonghi *et al*., 2012). Flavonols have the ability to reduce ROS in oxidative stress conditions including desiccation, which may explain their increase and role during postharvest withering (Rothschild and Mancinelli, 2001).

Structural genes involved in the anthocyanin biosynthetic pathway were also investigated during postharvest withering by Bonghi *et al*. (2012), and they were found to either undergo no changes in expression or to be down-regulated. Such was the case for

UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*) which regulates anthocyanin biosynthesis, as no change in the relative level of gene expression (Tonutti *et al*., 2004) or in transcript accumulation were observed (Bonghi *et al*., 2012). These observations are in contrast with the up-regulation of genes involved in anthocyanin production which has been observed in field cultivated grapes exposed to water-deficit (Castellarin *et al*., 2007; Grimplet *et al*., 2007). The literature offers conflicting information regarding the changes in concentration of anthocyanins in withered berries, with an increase found in 'Corvina' (Toffali *et al*., 2011) and in 'Sangiovese' (Bellincontro *et al*., 2004), a decrease in 'Cesanese' (Bellincontro *et al*., 2009), along with no significant changes reported in 'Pinot Noir' (Moreno *et al*., 2008) and 'Raboso Piave' (Bonghi *et al*., 2012) grapes. These discrepancies are likely to be a result of differences in experimental conditions and the intensity of dehydration, as well as genotypic variability between cultivars (Antelmi *et al*., 2010; Tonutti and Bonghi, 2013).

Stilbenes, such as resveratrol, have been shown to inhibit ROS production (Gresele *et al*., 2008) and represent another group of grape phenolpropanoids induced by biotic and abiotic stresses (Soleas *et al*., 1997). As previously discussed, an up-regulation in *STS* gene expression during withering has been demonstrated by Versari *et al*. (2001), and this observation has since been confirmed by Tonutti *et al*. (2004), Zamboni *et al*. (2008), Mencarelli *et al*. (2010) and Bonghi *et al*. (2012). However, this response appears to be modulated by drying temperature, with a down-regulation of *STS* observed by Mencarelli *et al*. (2010) at 30 ^{o}C conditions. Accumulation has been observed to begin at 7% cluster weight loss in slow dried samples, however no change in *STS* transcript abundance was detected until 17% water loss in fast dry conditions (Tonutti *et al*., 2004). The up-regulation

of *STS* which has been observed during drying, coupled with an increase in resveratrol concentration, suggests that resveratrol may play a role in modulating the postharvest stress response.

1.4.5 Dehydrins

The most common proteins that accumulate in plants in response to water or cold temperature stress are dehydrins (Close, 1997). Dehydrins comprise a large group of hydrophilic proteins found in a number of organisms including all higher plant species examined (Artlip and Wisnieski, 2002; Rorat, 2006). In *Arabidopsis,* six genes have been characterized (Puhakainen *et al*., 2004) and two similar dehydrin genes have been identified in both *Vitis vinifera* and *Vitis riparia* (Xiao and Nassuth, 2006). Dehydrins are believed to play a key role in promoting dehydration stress tolerance, although their exact mode of action is not understood. They are thought to prevent structural damage by stabilizing membranes and macromolecules, by maintaining essential activities of enzymes and by acting as scavengers of ROS (Close, 1997; Rorat, 2006; Xiao and Nassuth, 2006).

The induction of *DHN1a*, encoding dehydrin 1a, in vegetative tissues of *V. vinifera* and *V. riparia* has been observed in response to drought, cold temperature or ABA treatments (Xiao and Nassuth, 2006). An increase in *DHN1a* transcripts in *V. vinifera* has also been observed in shoot tips under water deficit and salinity stress (Cramer *et al*., 2007). Transcriptional profiling in 'Corvina' berries during postharvest withering has also demonstrated the up-regulation of *DHN1a* in both uncontrolled off-plant and on-plant withering, with a higher level observed in off-plant berries (Zamboni *et al*., 2008), which is consistent with its expected involvement in modulating the dehydration stress response.

1.4.6 Alcohol dehydrogenase

As berries begin to lose water during withering they experience shrinkage, which is an important consequence of the fruit drying process (Ramos *et al*., 2004). Shrinkage causes a modification of the size and often the shape of the fruit, and it is directly related to the water loss experienced (Ramos *et al*., 2004). A gradual shrinkage of the overall cell parameters was observed by Ramos *et al*. (2004) during the first stage of air drying in grape berry quarters. The change in cell structure experienced by berries during withering results in reduced intracellular spaces and the squeezing of cells, which alters membrane functionality (Ramos *et al*., 2004). This process is accompanied by an activation of LOX which has a role in altering membrane permeability, and finally leads to the formation of a barrier to gaseous diffusion (Chkaiban *et al*., 2007; Costantini *et al*., 2006; Kays, 1997). The restriction of gas diffusion generates a change in the gas concentration within cells and intracellular spaces, contributing to the creation of a partially modified atmosphere within the berries (Cirilli *et al*., 2012). This concentration change occurs at a time when oxygen demand is higher in the cells due to increased respiration associated with water stress, and ultimately contributes to the shift from aerobic to anaerobic metabolism which has been observed during withering, as well as the subsequent activation of ADH (Costantini *et al*., 2006; Cirilli *et al*., 2012; Franco *et al*., 2004).

ADH is an enzyme responsible for catalyzing the inter-conversion of acetaldehyde to ethanol, which under anaerobic conditions leads to fermentative metabolism (Tesnière and Abbal, 2009). It is known that ADH is induced in response to various types of stress in plants and it can be activated in *Arabidopsis* at a certain level of water loss (Dolferus *et al*., 1994). Its activity has also been shown to increase in potato during dehydration (Matton

et al., 1990), and a strong ADH activation has been demonstrated during intracellular anaerobic fermentation in grape (Flanzy *et al*., 1974). Six *ADH* genes have been discovered in *Vitis vinifera* (Tesnière and Abbal, 2009), with *VvAdh2* transcripts found to be the most abundant in berries among the isogenes (Tesnière and Verriès, 2000). As previously discussed, during postharvest withering in grapes, an increase in ADH activity associated with greater than 19.5% cluster weight loss in 'Malvasia' grapes has been demonstrated (Costantini *et al*., 2006), and similar results were found in 'Aleatico' berries at comparable drying parameters and weight loss levels (Cirilli *et al*., 2012).

The relative gene expression of *VvAdh1* and *VvAdh2* during postharvest withering in 'Aleatico' grape berries was investigated by Cirilli *et al*. (2012) under different drying conditions. No transcripts of *VvAdh1* were detected, however there was an increase in *VvAdh2* transcripts by 10% cluster weight loss in all temperatures, although the relative transcript amount and subsequent effects were different for each of the treatments. The highest relative gene expression was observed at 10 ^{o}C and 10% weight loss. In contrast to what has been observed for *VvAdh2*, in both slow and rapid drying conditions at 30% cluster weight loss Rizzini *et al*. (2009) found a decrease in accumulation in a putative *Adh7* transcript in 'Raboso Piave' berries. Taken together, the research conducted thus far supports the involvement of *VvAdh2* in the postharvest withering stress response in grape berries.

1.4.7 Osmoprotectants

A common response to osmotic stress and dehydration in plants is the accumulation of osmoprotectants or non-toxic, compatible osmolytes (Hare *et al*., 1998; Penna *et al*., 2006; Rontein *et al*., 2002). Although their actual physiological roles during water stress

remain a subject of some controversy, osmoprotectants are believed to function to stabilize molecules (Garg *et al*., 2002), and are also accumulated for the purposes of free radical scavenging, osmotic adjustment and water retention (Hussain, *et al*., 2012; Penna *et al*., 2006; Smirnoff, 1998). These compounds aid in osmotic adjustment through an influx of water into cells and/or a reduction in the amount of water lost from cells, which accompanies increases in their concentration (Hare *et al*., 1998). Compatible osmolytes include certain amino acids like proline, sugars such as trehalose and fructan, as well as sugar alcohols such as mannitol and sorbital (Garg *et al*., 2002; Hare *et al*., 1998; Penna *et al*., 2006).

There is some evidence for the involvement of osmoprotectants in the postharvest withering response in grape. As previously discussed, in 'Malvasia' berries, the dehydration stress response involves an accumulation of proline until a 11.7% weight loss, followed by a second significant rise in proline at 19% weight loss (Costantini *et al*., 2006). In addition, transcriptional profiling in 'Corvina' berries during postharvest drying has shown the up-regulation of a trehalose-phosphate phosphatase transcript during late withering (Zamboni *et al*., 2008). This enzyme is responsible for the last step in trehalose biosynthesis (Crowe *et al*., 2001). Trehalose is a disaccharide of glucose, which is accumulated in high amounts in many organisms capable of surviving complete dehydration, such as yeast (Crowe *et al*., 2001), and it has been shown to protect against dehydration stress at increased levels in *Escherichia coli* (Mig. 1895) Castellani and Chalmers 1919 (Garg *et al*., 2002). Trehalose has a unique reversible water-absorption feature (Hussain *et al*., 2012) and can stabilize proteins and membranes in the dry state (Crowe *et al*., 2001), forming a glass-like, high viscosity liquid structure (Goddijn *et al*.,

1999), which is thought to function to prevent the denaturation of biomolecules, thus allowing them to retain functionality upon rehydration (Fernandez *et al*., 2010). Trehalose does not seem to be accumulated in most plants to high levels, with the exception of a group of extremely desiccation-tolerant 'resurrection' plants that can recover from extreme dehydration (Goddijn *et al*., 1999; Penna *et al*., 2006). Besides resurrection plants, drought resistance has been associated with increased levels of trehalose in common bean (*Phaseolus vulgaris* L.) (Farias-Rodriguez *et al*., 1998), transgenic rice (Garg *et al*., 2002), tobacco (Zhao *et al*., 2000), tomato (Cortina and Culiáñez-Macia, 2005) and potato (Yeo *et al*., 2000).

Another group of osmoprotectants are sugar alcohols, which are believed to function by stabilizing macromolecules through maintaining an artificial sphere of hydration, due to their ability to imitate the structure of water (Penna *et al*., 2006). They may also have a role in the scavenging of ROS, which helps to protect against cell damage (Smirnoff, 1998). Sorbitol in particular is found most commonly in woody members of the Rosaceae, including all members of the genera *Malus* (apples), *Rosa* (Roses), *Prunus* (stone fruits) and *Pyrus* (pears), where it is the primary photosynthetic product (Loescher, 1987; Penna *et al*., 2006). Sorbitol is believed to function as an osmoprotectant, but its method of action and physiological role are not known (Ohta et al., 2005; Penna *et al*., 2006). Water stress has been associated with the accumulation of sorbitol in leaves of micropropagated apple (*Malus domestica* Borkh.) plants (Li and Li, 2005). In addition, transgenic *Nicotiana tabacum* L. plants which accumulate sorbitol also display increased drought tolerance (Sheveleva *et al*., 1998; Tao *et al*., 1995).

The up-regulation of a sorbitol-related enzyme during earlier stages of postharvest withering in 'Corvina' grape berries has been observed by Zamboni *et al*. (2008), and the up-regulation of a sorbitol transporter and dehydrogenase has also been reported in berries in the field after experiencing water-deficit (Grimplet *et al*., 2007). The up-regulation of both a sorbitol-related enzyme and of a trehalose-phosphate phosphatase during postharvest withering could positively affect the synthesis of sorbitol and trehalose respectively, and contribute to the water stress response in drying grape berries (Zamboni *et al*., 2008).

1.5 Conclusion and objectives

Recent research in transcriptional profiling of the postharvest withering of grape berries has been conducted primarily under uncontrolled, natural environmental conditions in Italy, using microarray and AFLP-TP approaches (Fasoli *et al*., 2012; Rizzini *et al*., 2009; Zamboni *et al*., 2010; Zamboni *et al*., 2008). Transcriptional profiling has revealed that the intensity and rate of postharvest dehydration affects the abundance of numerous gene transcripts in grape berry skins, with a higher number of differentially expressed genes associated with both a slower rate, as well as a more intense dehydration level (Rizzini *et al*., 2009).

The use of different rates of postharvest withering in grapes will each produce berries with their own unique compositions, which as a result will contribute to the production of diverse *appassimento* wine styles. However, a transcriptional profiling study comparing gene expression responses between controlled fast and slow withering has yet to be preformed, and previous gene expression studies have not employed the use of next generation sequencing technologies. The objective of this research was to use a completely

controlled, patented grape drying technology, in combination with RNA Sequencing (RNA-Seq), in attempts to characterize differential gene expression trends in both fast and slow *appassimento* methods, and to offer further insight into the postharvest withering stress response in grapes. The Vineland novel controlled drying technology can be used as a tool to create both fast and slow drying *appassimento* conditions, and gene expression information can provide a foundation for further research to investigate how similarities and differences between withering rates may be related to berry composition, and ultimately to the different *appassimento* wines produced.

Chapter 2. Materials and Methods

Contribution: The maintenance and monitoring of the postharvest withering process, as well as set-up of the grapes in the system was carried out by Kimberley Cathline. The design and construction of the experimental drying system, as well as arrangement of environmental conditions, was performed by Bernard Goyette (Vineland Research and Innovation Centre). All aspects of the sampling, berry compositional analysis, weight loss monitoring and RNA extraction, including sample preparation for RNA-Seq and RT-PCR, were carried out by Kimberley Cathline. RNA-Seq service for this research was carried out by the McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada). Bioinformatics was led by Travis Banks (Vineland Research and Innovation Centre). Processing of sequencing data was performed by Joseph O'Neill (Vineland Research and Innovation Centre) with direction and oversight from Travis Banks. Analysis of sequencing data was carried out by Travis Banks, Joseph O'Neill and Kimberley Cathline.

2.1 Plant material

Healthy red skinned grape clusters (*Vitis vinifera* L. 'Cabernet Franc') were carefully harvested by hand on October 30, 2014 from a commercial vineyard located in the Niagara Region appellation, Beamsville Bench Sub-Appellation, of Ontario, Canada. Harvest occurred when berries had reached the "Harvest-Ripe E-L 38" grapevine growth stage, according to the Modified Eichhorn-Lorenz (E-L) system for identifying grapevine growth stages (Coombe, 1995). At harvest, berries had an average total soluble solids (TSS) content of 21.7 ^{o}Brix.

2.2 Postharvest withering of 'Cabernet Franc' grapes

2.2.1 Withering treatments

High quality clusters with sound and healthy berries were harvested and gently placed into reusable plastic containers (RPC), containing openings in the sides and bottom (SmartCrate™, model IPL 6411, IPL Inc., Saint-Damien, Québec, Canada). The RPCs measured 60 cm long x 40 cm wide x 12 cm high (**Figure 2.1**) and are designed and optimised to allow air to circulate through and around a product (Vigneault and Goyette, 2002) when placed inside the proprietary ventilation technology described in section **2.2.2**. Each RPC contained an average of 8.6 kg of grape clusters. Grapes were stored under two different environmental conditions using a novel controlled drying technology designed at Vineland Research and Innovation Centre (Vineland, Ontario, Canada), in order to allow for two different withering rates:

a) Fast Withering, FW – Clusters were stored for 20 days at an average temperature and RH of 25 ^{o}C and 65% respectively, and an air speed of 0.2 m/s.

b) Slow Withering, SW – Clusters were stored for 132 days at an average temperature and RH of 6 ^{o}C and 77% respectively, and an air speed of 0.2 m/s.

Drying systems were monitored continuously using temperature and RH data loggers (HOBO® RX3000 and HOBO® U23 Pro v2, Onset Computer Corp., Bourne, Massachusetts, USA). The drying process was followed by monitoring TSS values almost daily in the fast withering treatment, and approximately on a weekly basis in the slow withering treatment. The drying process was considered complete once a target total soluble solids of 29 $\pm$ 1 ^{o}Brix was achieved.

Figure 2.1 Reusable plastic container used for harvest and postharvest withering of grapes, (SmartCrate™, model IPL 6411, IPL Inc., Saint-Damien, Québec, Canada).

2.2.2 Vineland's proprietary ventilation technology

A proprietary ventilation technology designed at Vineland Research and Innovation Centre (United States Patent Application Number - 14/032,619; Canadian Patent Application Number - 2,828,028) was used as an *appassimento* grape drying system in this experiment (**Figure 2.2**). This system consists of a plenum box equipped with a fan to create vertical air flow through the grapes, together with cooling and heating units used for environmental control of the temperature and RH. The fan component of the system creates suction in the plenum, which is connected to specially designed pallets. The pallet acts as a ducting system which transports the air that passes vertically through the grapes from the top to the bottom of the stacked RPCs wrapped in polyurethane film. Wrapped pallets are placed in a row on the side of the plenum, in such a way that the openings on the plenum fit the opening of the row of the pallets. Under these controlled conditions, air passes through the grapes to absorb humidity. The system is completely controllable and allows for tight modulation of the drying parameters.

A small-scale version of Vineland Research and Innovation Centre's (Vineland) drying system was used to conduct both fast and slow withering experiments described in this thesis. This system used the same proprietary ventilation technology as the commercial system and consisted of 10 individual RPCs stacked together on a plenum box, which was equipped with a fan (**Figure 2.3**). Cluster weight loss was monitored by weighing five separate RPCs periodically, from harvest through to completion of withering. Weights were measured using a scale accurate to 0.0005 kg with a 30 kg capacity (Ranger® RC30LS, Ohaus® Corp., Parsippany, New Jersey, USA), and weight loss was expressed as a percentage of original weight.

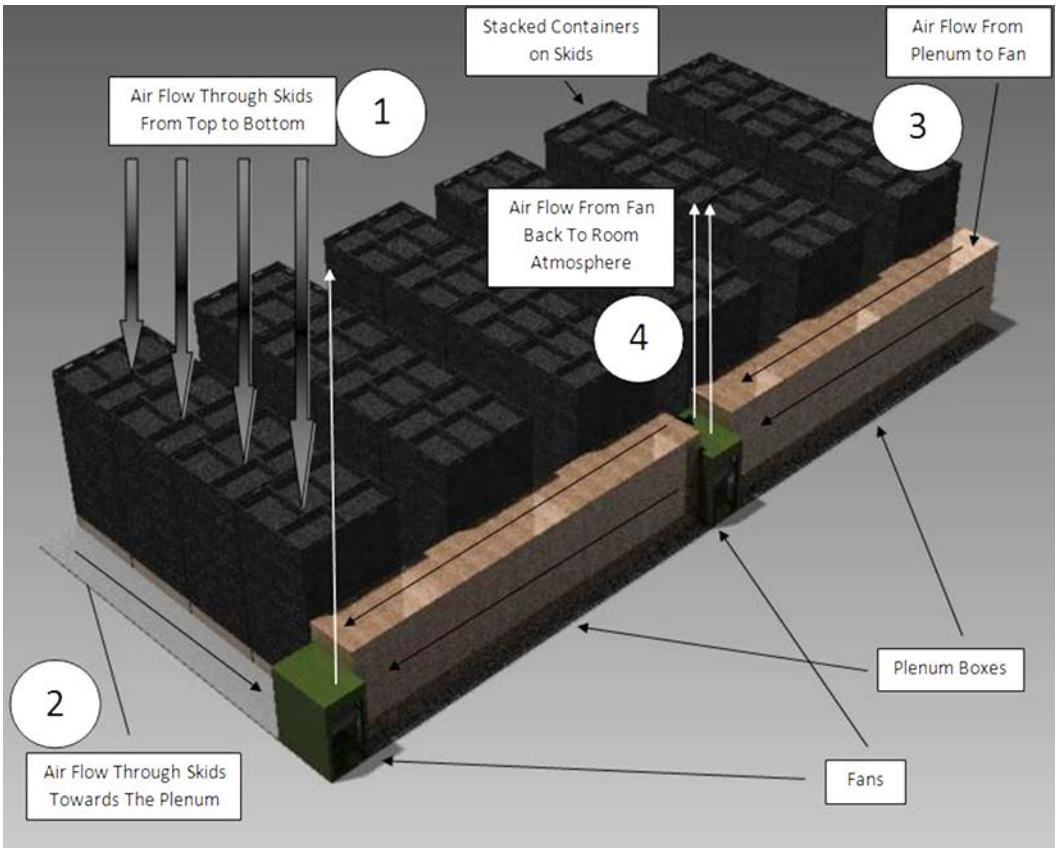


Figure 2.2 Schematic representation of Vineland Research and Innovation Centre's novel drying system (United States Patent Application Number - 14/032,619; Canadian Patent Application Number - 2,828,028).

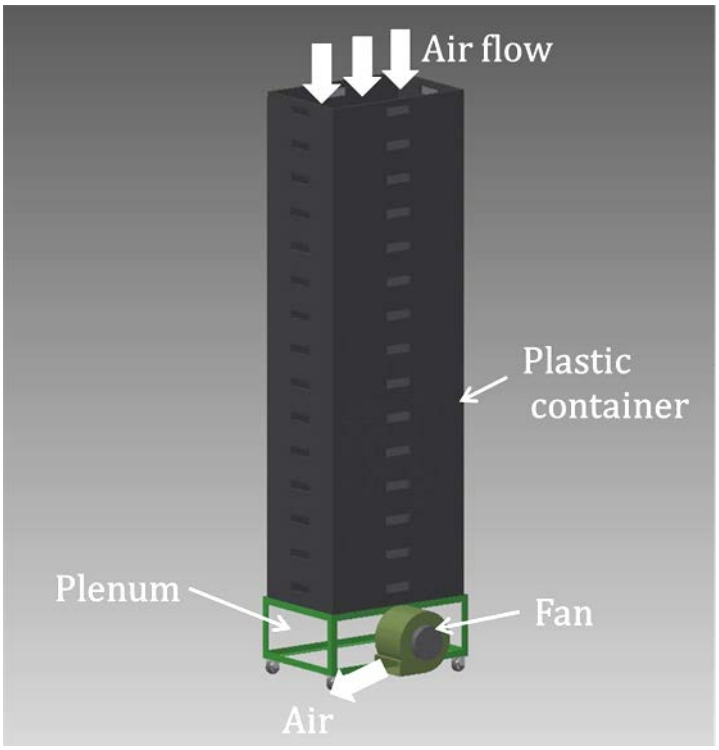


Figure 2.3 Schematic representation of a small-scale version of Vineland's novel drying system which was used to conduct both fast and slow withering in this experiment.

2.3 Sample collection and compositional analysis

Samples were collected at harvest (H), and at three time points (I, II, III) during both fast withering (FW) and slow withering (SW), with the last sampling point occurring on the final day of drying (**Table 2.1**). A total of 240 berries from the top, middle and bottom portions of 80 randomly selected clusters were collected for each sampling time point and withering treatment. Of these 240 berries, 180 berries, (taken from 60 clusters as described above), were collected by clipping the pedicel while leaving a portion attached to the berry, for immediate transfer and storage at –80 ^{o}C. The remaining 60 berries, (taken from 20 clusters as described above), were collected without pedicels to be homogenized in a blender, and the juice was filtered through two layers of cheesecloth for measuring the TSS (^{o}Brix) using a hand-held, temperature-compensating, digital refractometer (ATAGO® PAL-1, Atago Co., Ltd., Tokyo, Japan). Two independent measurements were made for each sample and averaged to obtain a final TSS (^{o}Brix) value. The pH and titratable acidity (g/L tartaric acid) of two ml of the filtered juice, diluted in 50 ml Milli-Q® water, was determined with an automatic titrator (Titrino 848, Metrohm AG, Switzerland), and titration was accomplished with 0.1 N sodium hydroxide to a pH 8.2 endpoint. Two independent titrations were conducted for each sample and averaged to obtain a final titratable acidity (g/L tartaric acid) value.

Table 2.1 Sampling time points during fast and slow postharvest withering of *Vitis vinifera* 'Cabernet Franc' grape berries.

Sampling time point	Days after harvest
Harvest: H	0
Fast withering: FW	
I	5
II	16
III	20
Slow withering: SW	
I	53
II	103
III	132

2.4 Statistical analysis of berry composition and percent weight loss

Berry compositional analysis values are expressed as the mean of two technical replications, comprised of 60 berries taken from the top, middle and bottom portions of 20 randomly selected clusters. Percent weight loss values are expressed as the mean of five replicates, each comprised of one separate RPC filled with clusters. Berry compositional analysis values, as well as percent weight loss measurements were subjected to analysis of variance (ANOVA) using Minitab 17 (Minitab, Inc., State College, Pennsylvania, USA). Differences were accepted as statistically significant when $P \leq 0.05$. Mean values were compared by Tukey's test ($\alpha = 0.05$) and significant difference attributed by letters.

2.5 Extraction of total RNA

Total RNA was extracted from grape berry tissue (skin and flesh) using PureLink® Plant RNA Reagent (Abion™ by Life Technologies™, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) according to the manufacturer's protocol (Publication

Number MAN0000243), with some modifications as follows. Success with this extraction protocol was achieved with careful optimization of the technique, including minimizing exposure to ribonucleases, which rapidly degrade RNA, by working on ice, carrying out steps rapidly, autoclaving reagents and glassware, baking mortars and pestles, and the use of clean gloves (Pilcher *et al*., 2007). This protocol was optimized after numerous repetitions to isolate total RNA from approximately 200 mg of tissue. Reagent volumes should be scaled appropriately if higher amounts of tissue are used. All microcentrifuge tubes and pipette tips were autoclaved and cooled completely prior to use. Mortars and pestles, along with tools such as tweezers and spatulas, were wrapped in tin foil and baked at 250^{o}C for 24 hours and cooled completely prior to use. All surfaces, pipettes and additional non-disposable items were sprayed and treated with RNaseZAP™ (Sigma-Aldrich Co., St. Louis, Missouri, USA) prior to commencing the extraction, and care was taken to periodically re-treat surfaces as needed throughout the process. Proper aseptic techniques were used throughout the extraction procedure, including the use of disposable gloves which were changed frequently. All solutions were prepared with sterile Milli-Q® water (EMD Millipore Corp., Billerica, Massachusetts, USA), autoclaved and cooled completely prior to use. All reagents and microcentrifuge tubes were cooled on ice prior to use and centrifugation was performed at 4^{o}C (Centrifuge 5418 R, Eppendorf AG, Hamburg, Germany). Each step was performed on ice where possible and unless specified otherwise.

For each time point, ten randomly selected berries from those stored at –80 ^{o}C were ground to a fine powder using a mortar and pestle in the presence of liquid nitrogen. Seeds and pedicles were carefully removed from frozen berries and discarded prior to grinding.

Approximately 200 mg of ground, frozen berry tissue was added to a pre-weighed 2.0 mL microcentrifuge tube. Remaining berry tissue was stored at –80 ^{o}C. To the microcentrifuge tube containing the frozen tissue, 1 mL of PureLink® Plant RNA Reagent was added, followed by briefly vortexing until the sample was fully suspended. The tube was then incubated at room temperature for 5 minutes by laying it horizontally to maximize surface area, during which time it was periodically shaken gently. After incubation, the solution was clarified by centrifugation for 5 minutes at 15,000 rpm and supernatant was transferred to a clean 2.0 mL microcentrifuge tube. The clarified extract was treated with 200 µL of 5 M sodium chloride and mixed by gently tapping the tube for 10 seconds. The sample was then treated with 600 µL of chloroform and mixed by gently inverting the tube repetitively for 10 seconds. The sample was then centrifuged for 30 minutes at 15,000 rpm to separate the mixture into two phases. The upper, aqueous phase was transferred to a clean 1.5 mL microcentrifuge tube, to which an equal volume of isopropyl alcohol was added, followed by gentle repetitive inversion for 10 seconds. The sample was then let stand on ice for 20 minutes to precipitate the RNA, followed by centrifugation at 15,000 rpm for 30 minutes. Supernatant was decanted gently and 1.5 mL of 75% ethanol was added to the pellet, followed by gentle repetitive inversion for 10 seconds. Sample was then pelleted again by centrifugation at 15,000 rpm for 5 minutes and supernatant gently decanted. The ethanol wash, subsequent centrifugation and decanting step were repeated three additional times. After the fourth time, the tube was then briefly centrifuged to collect the residual liquid, which was removed carefully with a pipette. RNA was resuspended by adding 20 µL of sterile Milli-Q® water to the tube and by gently tapping, in combination with carefully pipetting the liquid up and down over the pellet. Extractions were repeated three times for

each of the seven sampling time points, using 10 randomly selected berries each time, to generate three biological replicates and thus a total of 21 samples.

RNA quantity and quality were determined by spectrophotometer analysis through measuring the absorbance (NanoPhotometer® Classic, Implen U.S.A., Inc., Westlake Village, California, USA) at 230, 260 and 280 nm. The absorbance at 260 nm provides a measurement of quantity, the A_{260}/A_{280} (absorbance at 260 nm divided by the absorbance at 280 nm) and A_{260}/A_{230} ratios were calculated to determine the purity of the RNA sample. In addition, the integrity of RNA was verified by visualization of intact ribosomal bands on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis using 5 µL of RNA sample. Gels were visualized and photographed using a Molecular Imager® Gel Doc™ XR+ system with Image Lab™ Software (Bio-Rad Laboratories, Inc., Hercules, California, USA).

In order to look for the presence of full-length gene transcripts, RNA extraction samples from all seven time points underwent reverse transcription polymerase chain reaction (RT-PCR) using primers designed to amplify the open reading frame (ORF) of three different genes. Total RNA was used as a template for first-strand cDNA (complimentary deoxyribonucleic acid) synthesis using SuperScript™ II Reverse Transcriptase (Invitrogen™ by Life Technologies™, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) following the manufacturer's instructions. The presence of full-length transcripts of a phenylalanine ammonia lyase and a trehalose-phosphate phosphatase which have both shown up-regulation during withering of grape berries (Zamboni *et al*., 2008), as well as a lipid transfer protein that has been associated with water deficit in shoot tissue of grapes in the field (Cramer *et al*., 2007), were chosen to

investigate using PCR. Oligonucleotide primers were designed (Integrated DNA Technologies Inc., Coralville, Iowa, USA) to amplify the full-length ORF of the phenylalanine ammonia lyase (DFCI Grape Gene Index accession number TC150103), the trehalose-phosphate phosphatase (DFCI Grape Gene Index accession number TC67690) and the lipid transfer protein (NCBI gene accession number LOC100258473) from cDNA. The sequences of the primers were: phenylalanine ammonia lyase 5'-ATGGATGCAACGAACTGCCA -3' (forward) and 5'-CTAGCAGATTGGGAGAGGAGC -3' (reverse); trehalose-phosphate phosphatase 5'-ATGGATCTGAAGTCCAATCATTCT -3' (forward) and 5'-TTATAGTGCACTTGACTTCTTCCAC -3' (reverse); and lipid transfer protein 5'-ATGAATCCAGTGCCTTTCCCG -3' (forward) and 5'-TCATTTAGGGTGGACCAGGT -3' (reverse). The PCR reaction for all three genes was carried out using Taq DNA Polymerase (2X Taq FroggaMix, FroggaBio, Inc., Toronto, Ontario, Canada) under the following cycling conditions: initial denaturation at 94 ^{o}C for 3 minutes, then 34 cycles of 94 ^{o}C for 30 seconds, 52 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute, followed by a final extension period of 72 ^{o}C for 10 minutes (C 1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, California, USA). The presence of full-length gene transcripts was verified by visualization of bands on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis. A majority of samples showed clear bands corresponding to the expected ORF sizes of 2133 base pairs (bp) (phenylalanine ammonia lyase), 1158 bp (trehalose-phosphate phosphatase) and 1203 bp (lipid transfer protein). The same PCR reaction was also run using RNA in place of cDNA from all seven sampling time points to check for product amplification as an indication of

DNA contamination. No bands were present upon visualization on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis.

2.6 RNA sequencing

RNA-Seq service for this research was carried out by the McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada) using an Illumina® HiSeq 2500 ultra-high-throughput sequencing system using v4 chemistry (Illumina, Inc., San Diego, California, USA). The 21 total RNA samples (three biological replications of seven time points, **Table 2.1**) were prepared for shipping following McGill University and Génome Québec Innovation Centre's (Génome Québec) instructions (User Guide: Illumina® sequencing technologies, version 5.4). Once samples were received at Génome Québec, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Due to an unfamiliarity with *V. vinifera* and an absence of the required quality control metrics, Génome Québec was not able to provide confirmation that the RNA was intact and of good quality using their bioanalyzer method of analysis. Génome Québec also indicated a concern that the A_{260}/A_{230} ratios associated with the samples may indicate the presence of contamination that could possibly interfere with sequencing. The initial information provided by the spectrophotometer analysis, specifically the A_{260}/A_{280} ratios, along with the visualization of intact ribosomal bands after agarose gel electrophoresis (**2.5, Materials and Methods**) indicated that the RNA was intact and of good quality for all samples. However, without the ability of Génome Québec to confirm this information, and due to their concern surrounding the A_{260}/A_{230} ratios, it was important to attempt to further verify the positive quality observations made prior to

shipping the samples, and to address the concern regarding the A_{260}/A_{230} ratios, before proceeding with authorizing the RNA-Seq.

Modifications to the original RNA extraction protocol (**2.5, Materials and Methods**) were attempted, along with several additional techniques and tools, however all efforts resulted in much lower yields, as well as reduced A_{260}/A_{280} and A_{260}/A_{230} ratios. Following these unsuccessful attempts, a consultation was arranged with Norgen Biotek Corporation (Thorold, Ontario, Canada). Norgen Biotek Corporation employed the use of their Plant/Fungi Total RNA Purification Kit to extract RNA from ripe *V. vinifera* 'Cabernet Franc' berries that had not undergone postharvest withering. Unfortunately, the kit resulted in extremely low yields of RNA, making it an unacceptable choice for extraction for the purposes of this research. The kit was also unable to generate an increase in the A_{260}/A_{280} and A_{260}/A_{230} ratios and showed results that were comparable to what was achieved with the original RNA extraction protocol used in this experiment.

A final test was conducted before deciding whether or not to proceed with authorizing the RNA-Seq. In order to verify the presence of full-length gene transcripts in these specific samples, four sub-samples covering a range of RNA quantities and A_{260}/A_{280} and A_{260}/A_{230} ratios were chosen to undergo RT-PCR. These sub-samples were taken from the exact same RNA extractions of which samples were sent to Génome Québec. Total RNA was used as a template for first-strand cDNA synthesis using SuperScript™ II Reverse Transcriptase (Invitrogen™ by Life Technologies™, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) following the manufacturer's instructions, with a uniform 1.422 µg of RNA reverse-transcribed for each sample in a 20 µL reaction volume. The presence of full-length transcripts of the previously described lipid transfer protein (Cramer

et al., 2007) were chosen to investigate using PCR. The same oligonucleotide primers as previously described were used to amplify the ORF of the lipid transfer protein gene from cDNA (NCBI gene accession number LOC100258473). The PCR reaction was carried out using Taq DNA Polymerase (2X Taq FroggaMix, FroggaBio, Inc., Toronto, Ontario, Canada) under the following cycling conditions: initial denaturation at 94 ^{o}C for 3 minutes, then 34 cycles of 94 ^{o}C for 30 seconds, 52 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute, followed by a final extension period of 72 ^{o}C for 10 minutes (C 1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, California, USA). The presence of full-length gene transcripts was verified by visualization of bands on a 1% agarose gel stained with ethidium bromide, following gel electrophoresis. All four samples showed clear bands corresponding to the expected ORF size of 1203 bp.

The total RNA quality and quantity based on the A_{260}/A_{280} ratios, the detection of intact ribosomal bands after agarose gel electrophoresis and the ability to amplify full-length gene products following RT-PCR triggered an authorization to Génome Québec to proceed with the RNA-Seq of the provided samples. At Génome Québec, cDNA libraries were created from 250 ng of total RNA using the Illumina® TruSeq® Stranded mRNA Sample Preparation Kit (Illumina, Inc., San Diego, California, USA), as per the manufacturer's recommendations, which included mRNA enrichment using poly-T oligo attached magnetic beads using two rounds of purification, mRNA fragmentation and priming for cDNA synthesis using random hexamers. Second strand cDNA synthesis involved the incorporation of dUPT, in order to quench the second strand during amplification. Following double stranded cDNA synthesis a single 'A' nucleotide was added to the 3' ends of the fragments to allow for ligation of the sequencing primers, which

had a corresponding 'T' nucleotide overhang on their 3' end. Fragments were then enriched using PCR, followed by hybridization to flow cells for sequencing. Libraries were sequenced with an Illumina® HiSeq 2500 ultra-high-throughput sequencing system using v4 chemistry (Illumina, Inc., San Diego, California, USA), following the manufacturer's instructions. Prior to sequencing, solid-phase bridge amplification was used to generate clusters of up to 1,000 copies of each single fragment in close proximity to each other. An Illumina® PhiX library spike-in was used as a control and mixed with the sample libraries at a level of one percent (Illumina, Inc., San Diego, California, USA). Four fluorescently-labelled reversible terminator nucleotides were used to sequence clusters in parallel and 125-bp paired-end sequences were generated. Illumina® Real-Time Analysis (RTA) software, Version 1.18.64, (Illumina, Inc., San Diego, California, USA) calculated the number of reads per sample and the average *phred* quality score (*Q*-score) for each sample (Ewing and Green, 1998).

2.7 Sequencing data processing and analysis

For pre-analysis and quality control, the sequence reads from all 21 samples were analyzed using FASTQC software, Version 0.11.5, (Babraham Bioinformatics, Cambridge, UK) as an initial step. For each sample, forward and reverse paired-end reads were merged using PEAR (Paired-End reAd mergeR) software, Version 0.9.6 (Zhang *et al.*, 2014). Both merged reads and reads that could not be merged were kept for downstream analysis, but they were retained separately. Illumina® adapter sequences were clipped and low-quality bases/reads were removed from sequence reads in each sample (minimum *Q*-score 13, minimum read length 25 bp) using FaQCs software, Version 1.34 (Lo and Chain, 2014). For overall alignment rates, raw sequence reads were mapped to the current National

Centre for Biotechnology Institute (NCBI) *Vitis vinifera* Annotation Release 101 genome (http://www.ncbi.nlm.nih.gov/genome/annotation_euk/Vitis_vinifera/101/) using Bowtie software, Version 1.1.2 (Langmead *et al*., 2009). For differential gene analysis, sequence reads were aligned to protein-coding regions against the *V. vinifera* reference genome using STAR (spliced transcripts alignment to a reference) software, Version 2.5 (Dobin *et al*., 2013). For each sample, the STAR aligner outputs a reads-per-gene file. Using a custom script, these multiple reads-per-gene files were merged into a master reads-per-gene matrix, where columns are samples and rows are genes. This matrix was imported into the R software environment, Version 3.3.1 (The R Foundation, www.r-project.org). Analysis of differential gene expression was conducted by comparing each sampling time point to the harvest time point in R with the *limma* package, Version 3.28.21 (Ritchie *et al*., 2015). Based on the distribution of P-values for unfiltered reads-per-gene data, a cut-off was calculated which allowed genes with very low reads to be removed from each differential gene expression analysis. The threshold for a differentially expressed gene was set as: false discovery rate (FDR) $\leq$ 0.01, $\log_2$ fold change (lfc) $\geq$ 1. Principal component analysis (PCA) was performed using the R software environment. Comparison of expression patterns for genes of interest over time was performed in the Perl software environment, Version 5.20 (www.perl.org). The numerical identifiers for the genes of interest were first identified in the reference genome (NCBI *Vitis vinifera* Annotation Release 101) and then these identifiers were used to locate the same genes within withering samples to follow general expression over time.

Gene ontology (GO) identifiers were assigned to each of the genes in the NCBI *Vitis vinifera* Annotation Release 101 genome by the use of the Basic Local Alignment

Search Tool (BLAST) method, in combination with the *Vitis vinifera* Genoscope database (http://www.genoscope.cns.fr/externe/Download/Projets/Projet_ML/data/12X/annotation/Vitis_vinifera_peptide.fa.gz). The top hit from the Genoscope database was used to identify the GO identifier for the associated gene in the NCBI genome, as long as the top hit had a P-value < 0.0001. In cases where the top hit had a P-value greater than the established threshold, no GO identifier was assigned. Distribution of DE genes by GO term was analyzed using agriGO (Du *et al*., 2010). Using this web-based tool, significantly enriched GO terms represented within lists of Genoscope GO identifiers were determined by means of Fisher statistical test with a significance level of $P \leq 0.05$ using the following settings: singular enrichment as the analysis tool, *Vitis vinifera* as the species and the suggested background as the reference. Significantly overrepresented GO terms associated with $\geq$ 5 percent of the total DE expressed genes analyzed were included in the results.

Botrytis cinerea Pers. (1794) alignment rates were determined by aligning raw reads to the latest version of the *Botrytis cinerea* B05.10 reference genome in NCBI's Genbank (Amselem *et al*., 2011; Staats and van Kan, 2012). Alignment and statistical analysis was performed using Bowtie software, Version 1.1.2 (Langmead *et al*., 2009). Alignment rates were subjected to ANOVA ($P \leq 0.05$). Mean values were also compared by Tukey's test ($\alpha = 0.05$).

Chapter 3. Results

3.1 Both a slow *appassimento* withering process of over 100 days and a fast process of 20 days are achievable using a completely controlled novel drying system

Vineland's proprietary ventilation technology was able to successfully dry 'Cabernet Franc' grapes at both a fast withering rate, which lasted 20 days, as well as at a slow withering rate, lasting 132 days. Berry quality remained high throughout the drying process and clusters did not show any noticeable development of fungal growth (**Figure 3.1**). Cluster weight loss at the end of the slow drying period was 40.7 percent (**Figure 3.1**), which is consistent with what is achieved in Italy through the use of the traditional uncontrolled *appassimento* technique (Paronetto and Dellaglio, 2011; Tonutti *et al*., 2004). With respect to fast drying, a 31.2 percent weight loss was achieved (**Figure 3.1**).

3.2 Grape berry composition changes over the course of the postharvest drying period and is affected by the rate and intensity of the withering treatment

The withering process was monitored by following the average percent cluster weight loss and the total soluble solids content (^{o}Brix). The drying process was considered complete once a target total soluble solids of 29 $\pm$ 1 ^{o}Brix was achieved. Withering was completed after 20 days under the fast drying conditions, and grape clusters lost an average of 31.2 percent of their initial weight. Those clusters dried under the slow withering conditions lost 40.7 percent of their initial weight by the completion of the process, following 132 days of drying (**Table 3.1**). Water loss was accompanied by increases in the sugar concentration and total soluble solids from 21.7 ^{o}Brix to 28.2 and 29.8 ^{o}Brix respectively, as a result of fast and slow withering treatments (**Table 3.1**).

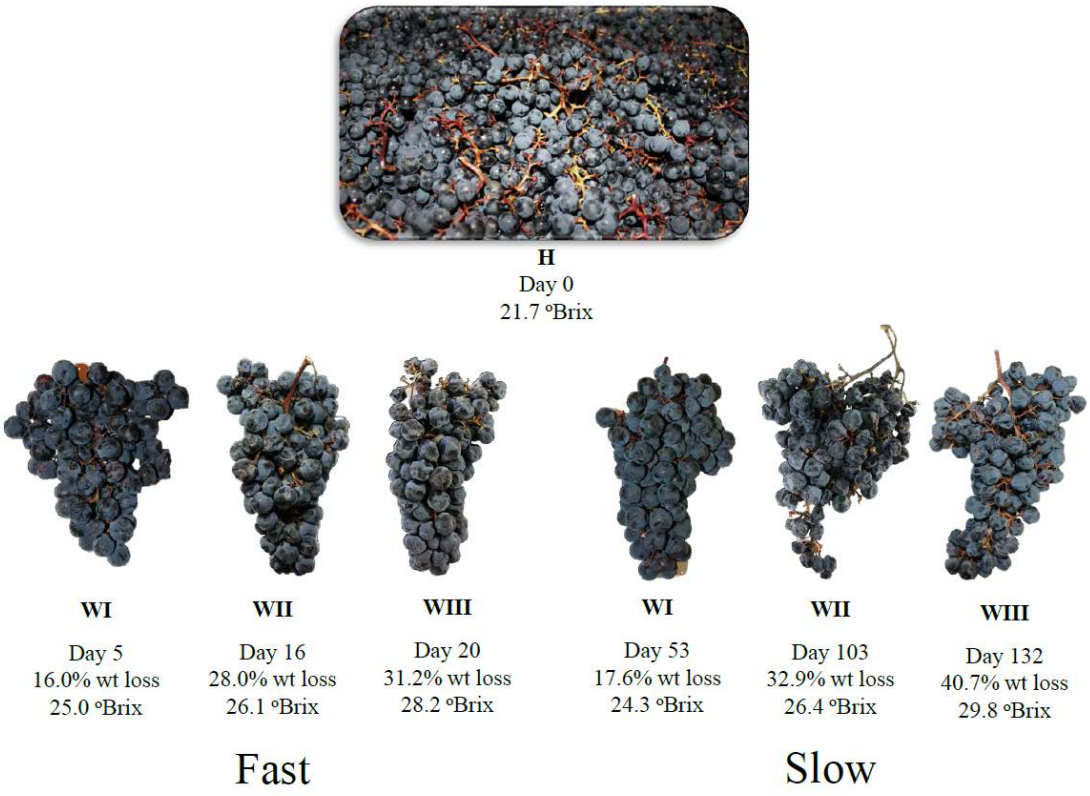


Figure 3.1 Representative clusters of *Vitis vinifera* 'Cabernet Franc' from each sampling time point across the postharvest withering period, demonstrating berry condition and appearance over time.

Table 3.1 Effect of postharvest withering rate and drying intensity on the total soluble solids, titratable acidity and pH of grape berry juice, as well as on average percent cluster weight loss in *Vitis vinifera* 'Cabernet Franc'.

Sampling time point	Total soluble solids (°Brix)	Titratable acidity (g/L)	pH	Weight loss (%)
Harvest	21.7[z] g	7.400 bc	3.61 a	0 d
Fast Dry				
WI	25.0 e	7.793 a	2.97 a	16.0 c
WII	26.1 d	6.994 de	3.70 a	28.0 b
WIII	28.2 b	6.750 e	3.71 a	31.2 b
Slow Dry				
WI	24.3 f	7.020 de	3.82 a	17.6 c
WII	26.4 c	7.583 ab	3.55 a	32.9 b
WIII	29.8 a	7.193 cd	3.73 a	40.7 a

[z]Values are treatment means, n = 2, except for weight loss where n = 5. Means followed by a different letter, within columns, are significantly different according to Tukey's test ($\alpha = 0.05$) at $P \leq 0.05$.

Titratable acidity (TA) increased significantly from 7.400 to 7.793 g/L by 16 percent weight loss in the fast withering treatment (**Table 3.1**). This was followed by a significant drop in TA to 6.994 g/L once reaching 28 percent weight loss, which remained at a statistically similar level until the end of drying at 31.2 percent weight loss. For the slow withering, at 17.6 percent weight loss the TA had dropped significantly from 7.400 to 7.020 g/L, which was a level statistically similar to that found at the end of the fast drying treatment. This drop was followed by a significant increase in TA at 32.9 percent weight loss to 7.583 g/L, which was however not significantly different from the level found in harvest samples. Finally, by 40.7 percent weight loss the TA dropped to 7.193 g/L, which was not significantly different from the level observed at SWI. The first two sampling time points (WI and WII) had statistically similar levels of weight loss in both the fast and slow withering treatments, however both the TA values were significantly different from each

other when comparing both drying conditions. Overall, the TA was lower than harvest values by the end of both fast and slow withering, however this drop was only statistically significant in the fast treatment.

In order to distinguish between the effects of concentration due to water loss and those changes which may be due to metabolic processes, the TA values at harvest and throughout the postharvest drying process were normalized to total soluble solids content, and the adjusted values are presented in **Table 3.2**. Normalized TA values demonstrate a reduction over the course of both fast and slow withering treatments. Statistically similar TA concentrations were observed at the end of drying when comparing the two treatments, and these concentrations were significantly different from the harvest value for both fast and slow withering.

Table 3.2 Effect of postharvest withering rate and drying intensity on the titratable acidity of grape berry juice in *Vitis vinifera* 'Cabernet Franc', normalized to total soluble solids content.

Sampling time point	Total soluble solids (°Brix)	Titratable acidity (g/L)/(°Brix)
Harvest	21.7[z] g	0.338 a
Fast Dry		
WI	25.0 e	0.312 b
WII	26.1 d	0.268 d
WIII	28.2 b	0.240 e
Slow Dry		
WI	24.3 f	0.290 c
WII	26.4 c	0.288 c
WIII	29.8 a	0.242 e

[z]Values are treatment means n = 2. Values followed by a different letter, within columns, are significantly different according to Tukey's test ($\alpha = 0.05$) at $P \leq 0.05$.

3.3 Extraction of total RNA

While A_{260}/A_{230} values remained low after performing several extractions using modified protocols, the protocol used (**2.5, Materials and Methods**) did not interfere with the ability to successfully perform RNA-Seq at any of the sampling time points. The optimized extraction method produced high quality total RNA from all sampling time points (**Table 3.3**). Intact ribosomal RNA bands for all time points were clearly observed after staining of agarose gels with ethidium bromide following gel electrophoresis. These results were also observed for ribosomal RNA found in the slow withering process which lasted 132 days with clusters sustaining a 40.7 percent weight loss. **Figure 3.2** shows a representative gel electrophoresis image demonstrating intact ribosomal bands which were visualized for seven different samples. The results displayed in **Figure 3.2** are representative of the intact ribosomal bands which were observed for all 21 samples extracted.

Table 3.3 Yields and purity absorbance ratios of total RNA isolated from *Vitis vinifera* 'Cabernet Franc' berry tissue from harvest (H), fast withering (FW) and slow withering (SW) sampling time points using the described protocol (**2.5, Materials and Methods**).

Sample name	Concentration[z] (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
H			
Rep 1	332	1.565	0.435
Rep 2	335	1.696	0.521
Rep 3	170	1.700	0.407
FWI			
Rep 1	258	1.635	0.422
Rep 2	318	1.587	0.412
Rep 3	260	1.891	0.168
FWII			
Rep 1	120	1.655	0.236
Rep 2	478	1.540	0.424
Rep 3	235	2.043	0.153
FWIII			
Rep 1	278	1.734	0.468
Rep 2	535	1.574	0.294
Rep 3	125	1.667	0.368
SWI			
Rep 1	195	1.696	0.268
Rep 2	578	1.471	0.451
Rep 3	218	1.706	0.401
SWII			
Rep 1	470	1.554	0.450
Rep 2	475	1.570	0.477
Rep 3	200	1.818	0.293
SWIII			
Rep 1	392	1.635	0.269
Rep 2	602	1.535	0.459
Rep 3	392	1.635	0.269

[z]Extractions were preformed with approximately 200 mg of berry tissue (see **2.5, Materials and Methods**)

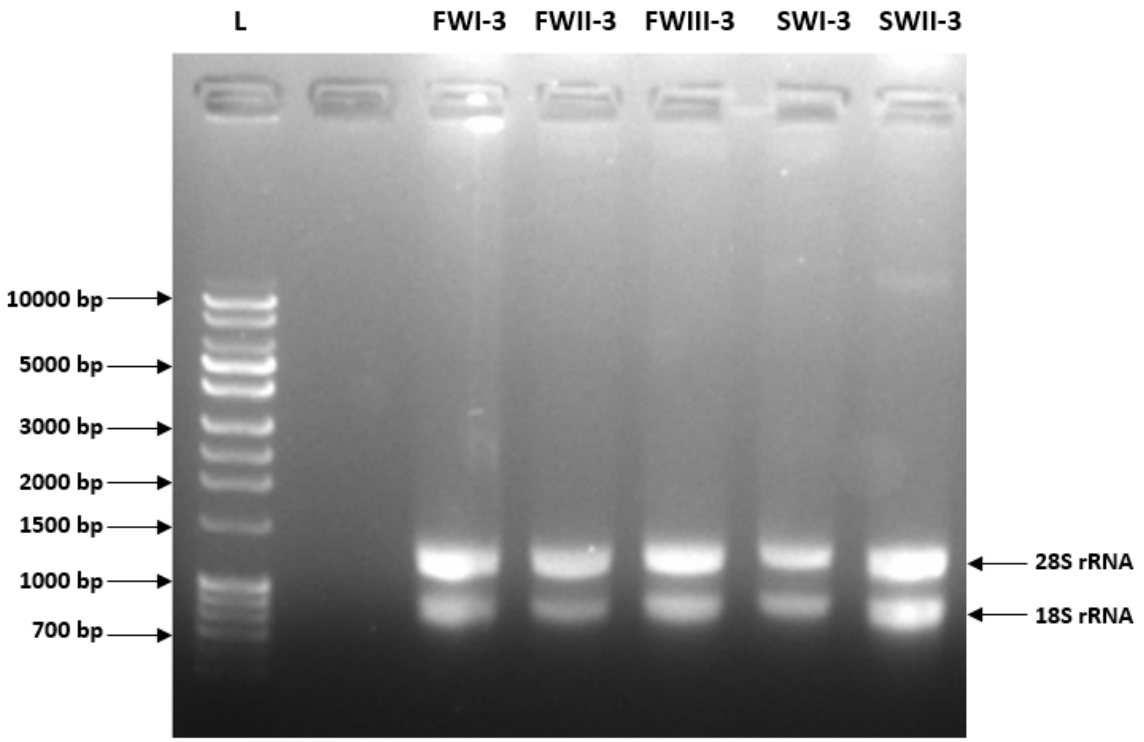


Figure 3.2 Gel electrophoresis image stained with ethidium bromide demonstrating ribosomal bands from total RNA isolated from *Vitis vinifera* 'Cabernet Franc' berry tissue at seven representative time points after undergoing postharvest withering. Extraction was completed using the procedure described in **2.5, Materials and Methods**. *Lane L* is HighRanger Plus 100bp DNA Ladder (Norgen Biotek Corporation, Thorold, Ontario, Canada); *FWI-3* is fast withering time point one, replicate #3; *FWII-3* is fast withering time point two, replicate #3; *FWIII-3* is fast withering time point three, replicate #3; *SWI-3* is slow withering time point one, replicate #3; *SWII-3* is fast withering time point two, replicate #3.

3.4 RNA Sequencing

RNA-Seq was successfully carried out for all 21 withering berry samples, including all time points and biological replicates. A principal component analysis (PCA) was conducted using RNA-Seq data in order to visualize the relationship between samples and their replicates (**Figure 3.3**). Principal component 1 (PC1) accounted for 82 percent of the total variance and principal component 2 (PC2) accounted for 11 percent.

The three time points for slow withering clustered together and the same was observed for fast withering. Both slow and fast clearly separated out from each other along PC1, with fast withering closest to the harvest samples. As compared to replicates at other time points, the H, FWI, FWII and SWI time points had the tightest grouping and thus the least amount of variance between them. The SWII and SWIII time points had the greatest variance between their replicates and they were not distinct from each other. FWIII was also not completely distinct from FWII and the replicates from both time points were quite close to each other. Overall the H and FWI time points were the most distinctly separated. An investigation into the number of differentially expressed (DE) genes in WIII as compared to WII revealed that when comparing differential regulation between only these two time points, there were no genes considered DE between them for both S and F treatments. When comparing differential regulation between only the WI and WII time points, the number of DE genes in SWII when compared to SWI was 19, and the number in FWII when compared to FWI was 376.

Each sample generated high quality reads, which is demonstrated through the average *phred* quality scores (*Q*-scores) that were assigned (**Table 3.4**) (Ewing and Green, 1998). Not only were reads of high quality, but the counts for the number of reads were

also well above the minimum recommendations for optimizing sequencing depth in differential gene expression experiments (**Table 3.4**) (Liu *et al*., 2014). Overall alignment rates to the *V. vinifera* reference genome (NCBI *Vitis vinifera* Annotation Release 101) ranged between approximately 58 to 75 percent (**Table 3.4**). All other subsequent quality control tests conducted using FASTQC software confirmed that data from all samples was of high quality and suitable for use in differential gene expression analysis.

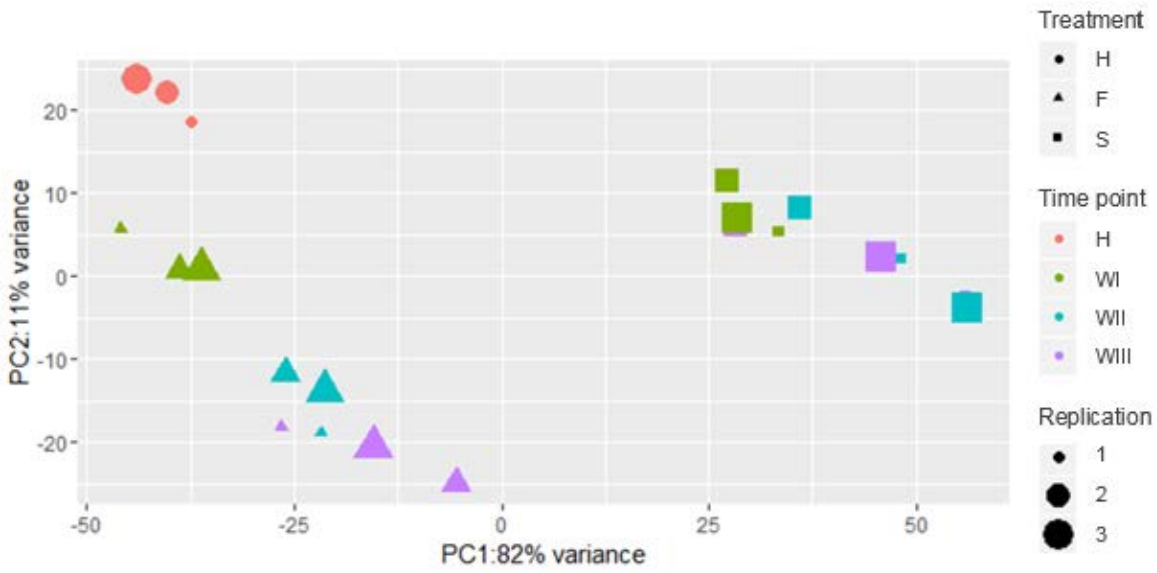


Figure 3.3 Principal component analysis plot showing RNA-Seq gene expression data for 21 samples of *Vitis vinifera* 'Cabernet Franc' berries taken at harvest (H) and over time during fast (F) and slow (S) postharvest withering. The first two principal components accounting for the greatest variation (PC1 and PC2) are plotted, with PCI explaining 82 percent and PC2 explaining 11% of the proportion of variance, which is indicated along the axis labels. Samples were collected at three time points during withering (I, II, III) and three biological replicates (1, 2, 3) underwent RNA-Seq for each time point.

Table 3.4 Average *phred* quality score (*Q*-score), number of reads, percent alignment to the *Vitis* reference genome and number of reads aligned to protein-coding regions (unique, non-duplicated regions) for each sample submitted for RNA-Seq. Total RNA was isolated from *Vitis vinifera* 'Cabernet Franc' berry tissue at harvest (H), fast withering (FW) and slow withering (SW) sampling time points.

Sample name	Average *phred* quality score	Number of raw reads[x]	Alignment rate[y] (%)	Number of protein-coding reads[z]
H				
Rep 1	36	43,768,294	64.81	29,989,961
Rep 2	36	34,671,168	74.99	30,617,118
Rep 3	36	37,986,016	75.08	35,455,990
FWI				
Rep 1	36	39,420,143	65.65	27,227,605
Rep 2	36	43,335,324	65.57	19,761,743
Rep 3	35	35,192,870	67.08	14,106,218
FWII				
Rep 1	35	36,640,903	66.5	33,797,045
Rep 2	35	35,999,756	62.52	18,139,977
Rep 3	36	40,389,093	58.41	7,363,466
FWIII				
Rep 1	35	31,261,710	70.73	25,632,911
Rep 2	35	40,278,191	62.56	14,710,881
Rep 3	35	43,808,556	63.37	32,624,909
SWI				
Rep 1	36	35,885,189	73.29	28,800,546
Rep 2	35	31,381,475	73.02	14,139,160
Rep 3	35	40,796,558	65.92	22,623,939
SWII				
Rep 1	36	30,265,821	69.71	26,983,111
Rep 2	35	52,020,918	66.98	42,366,394
Rep 3	36	32,665,595	66.37	27,595,549
SWIII				
Rep 1	36	33,316,131	69.06	24,436,356
Rep 2	35	32,649,645	68.14	29,444,803
Rep 3	35	43,525,087	62.14	24,769,026

[x]125-bp paired-end sequences
[y]Aligned with Bowtie software
[z]Aligned with STAR software to NCBI *Vitis vinifera* Annotation Release 101

3.5 Analysis of differential gene expression

Analysis of differential gene expression at each time point, as compared to harvest, demonstrated an overall increase in the total number of DE genes as dehydration intensity increased from WI to WII, in both fast and slow drying (**Figure 3.4**). The number of DE genes further increased at the FWIII time point, while there was a slight drop at SWIII. Overall, the slow withering process had a much higher number of DE genes at every time point as compared to fast withering. The highest number of DE genes in the fast withering samples was 3,082 at FWIII, and in the case of slow withering it was 6,772 at SWII. There were a total of 3,856 DE genes that were common across all three slow withering time points (**Figure 3.5A**), of which 2,156 were up-regulated and 1,700 down-regulated (**Figure 3.4**). With respect to fast withering, there were a total of 1,172 DE genes that were common across all three time points (**Figure 3.5B**), of which 534 were up-regulated and 638 down-regulated (**Figure 3.4**). Those DE genes which were commonly regulated represented a minimum of 56 or 38 percent of the total at any given time point, for slow and fast withering respectively. The proportion of common DE genes that were up-regulated was always greater than those that were down-regulated for all slow withering time points, and the opposite was true for all fast withering time points (**Figure 3.4**).

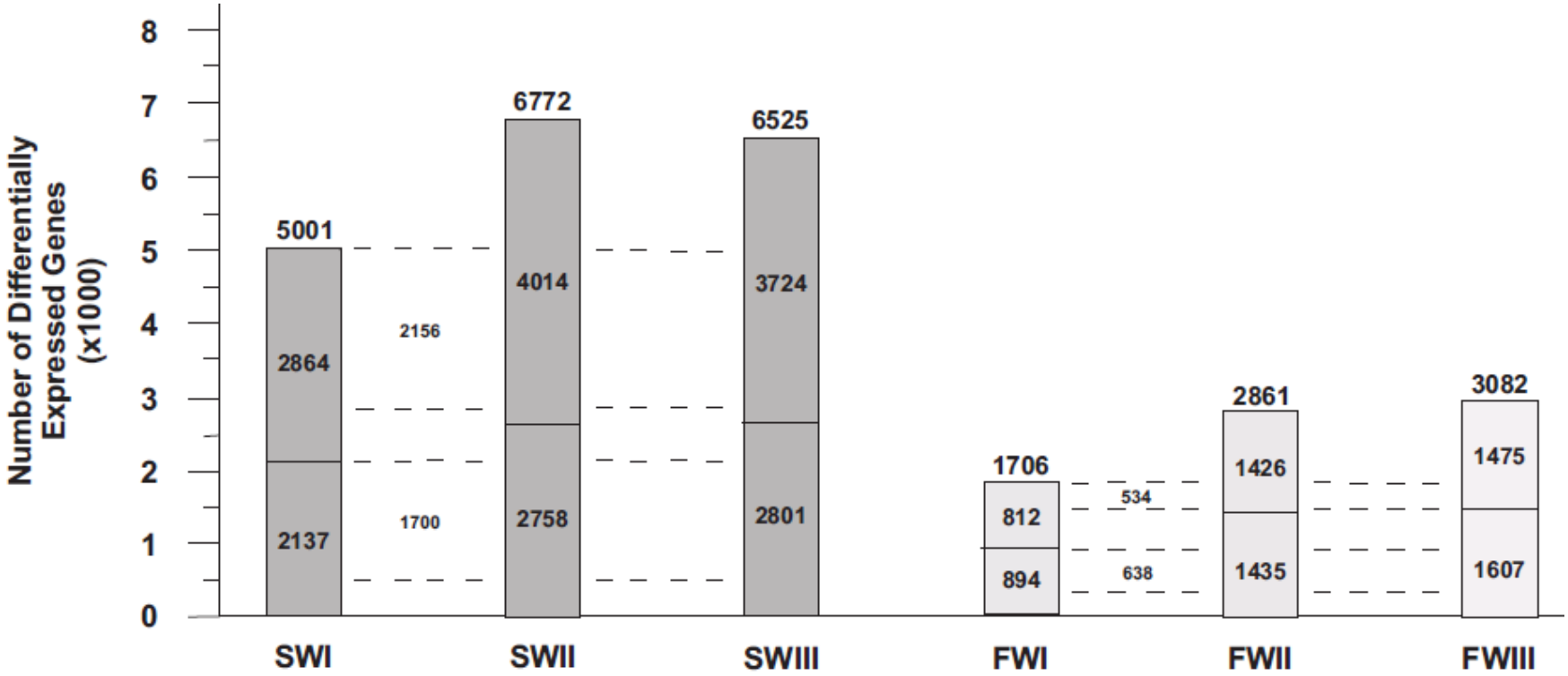
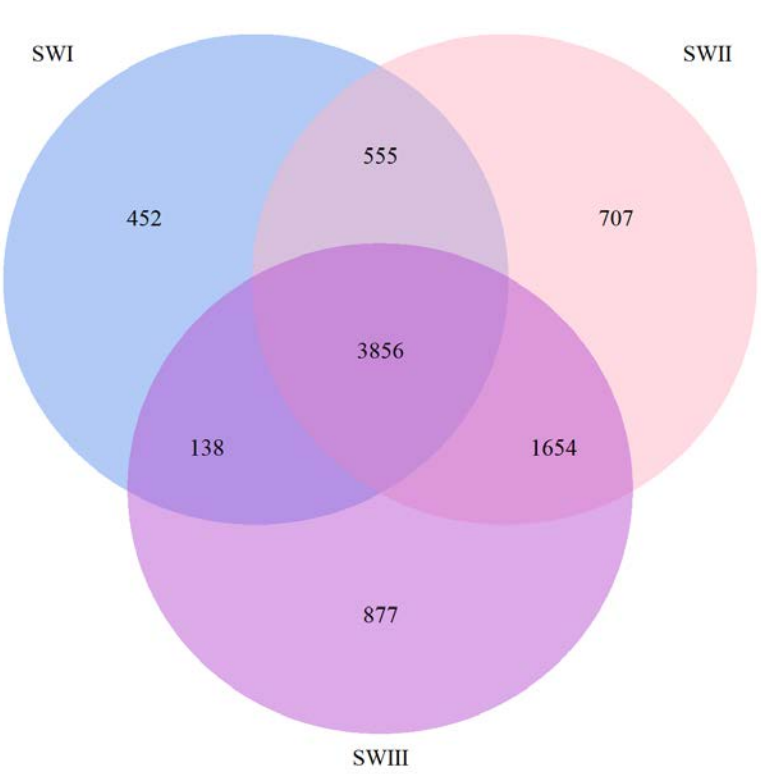


Figure 3.4 Differentially expressed genes in *Vitis vinifera* 'Cabernet Franc' grape berries over time, following different postharvest withering rates and intensities. Numbers associated with grey bars represent the total differentially expressed genes (above bars), along with the amount of up- (top section inside bars) and down- (bottom section inside bars) regulated genes identified by RNA-Seq analysis in the six samples, as compared to harvest (H). Numbers of up- and down-regulated genes showing the same regulation at all three slow (S) or fast withering (F) time points are indicated within the dotted lines.



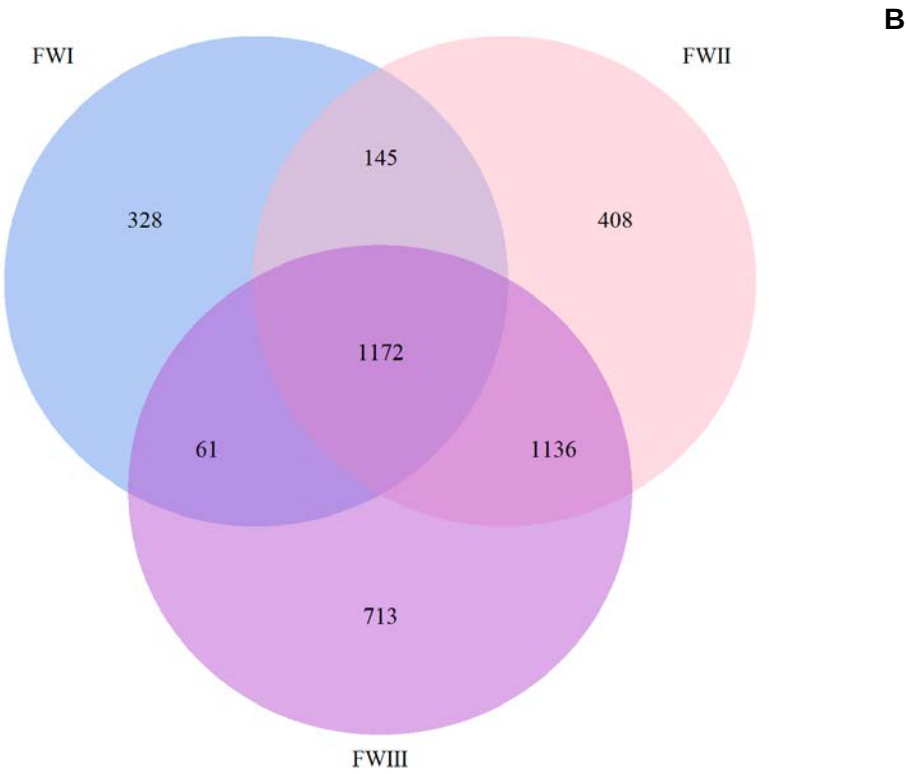


Figure 3.5 Venn diagrams of similarly regulated differentially expressed (DE) genes as compared to harvest in *Vitis vinifera* 'Cabernet Franc' berries across all time points (WI, WII, WIII) for (**A**) slow (S) and (**B**) fast (F) withering. Numbers represent DE genes and those associated with areas where circles overlap represent the total number of common DE genes at the corresponding time points.

The number of DE genes at WI, WII and WIII which were similarly regulated in both the fast as well as the slow withering are shown in **Figure 3.6**. This number increased along with withering intensity and weight loss from WI through WIII. For the individual time point comparisons, the amount of commonly up-regulated genes was always greater than the number of commonly down-regulated genes. The number of those similarly regulated DE genes which were common to all six withering time points are shown in **Figure 3.7**. There were 432 DE genes which were similarly regulated in all six withering time points, regardless of drying rate or intensity. The proportions of the common DE genes which were up- or down-regulated for all time point comparisons are shown in **Table 3.5**.

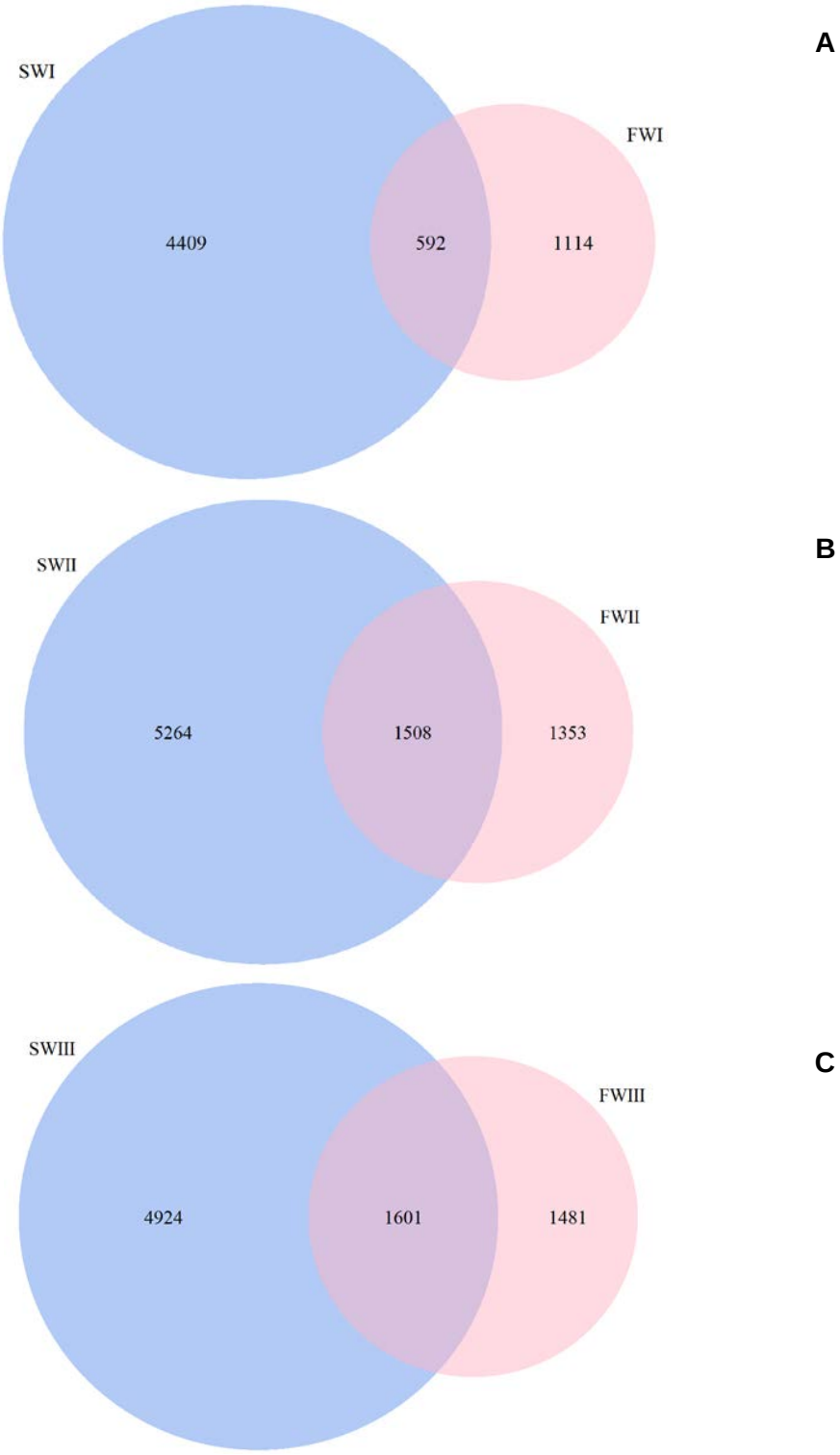


Figure 3.6 Venn diagrams of similarly regulated differentially expressed (DE) genes as compared to harvest in *Vitis vinifera* 'Cabernet Franc' berries at each time point for slow (S) and fast (F) withering. (**A**) DE genes at WI, (**B**) at WII and (**C**) at WIII time points. Numbers represent DE genes and those associated with areas where circles overlap represent the total number of DE genes that are common to both F and S at that particular time point.

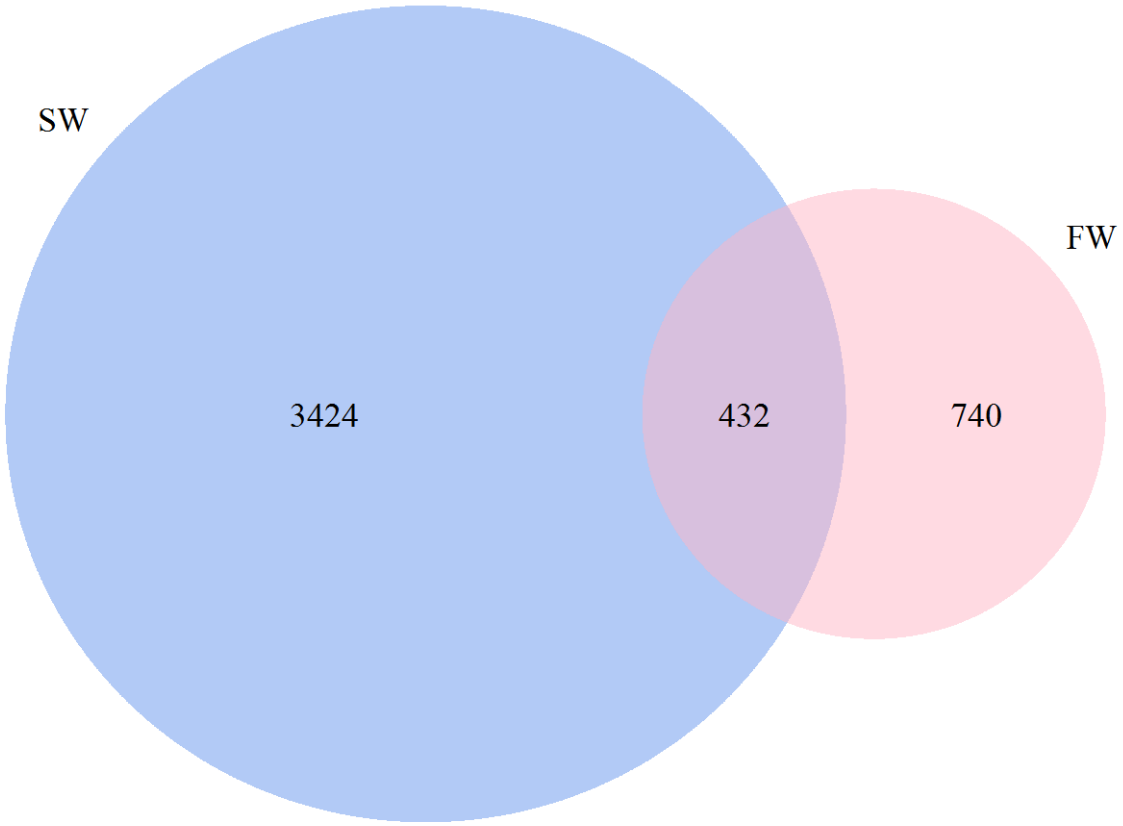


Figure 3.7 Venn diagram of similarly regulated differentially expressed (DE) genes as compared to harvest in *Vitis vinifera* 'Cabernet Franc' berries which are common to slow (S) and fast (F) withering. Numbers associated with the blue circle represent DE genes similarly regulated in SWI, SWII and SWIII, and those associated with the pink circle are similarly regulated in FWI, FWII and FWIII. Those associated with the area where circles overlap represent the total number of DE genes that are commonly regulated at all six sampling time points regardless of drying rate or intensity.

Table 3.5 Numbers of differentially expressed (DE) genes in common and showing similar regulation when comparing various combinations of sampling time points for slow (S) and fast (F) postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries. These numbers were determined from the analysis of RNA-Seq data and are further broken down into the number of up- and down-regulated genes.

Comparisons	Number of common DE genes	Number of up-regulated DE genes	Number of down-regulated DE genes
SWI, FWI	592	310	282
SWII, FWII	1508	826	682
SWIII, FWIII	1601	836	765
SWI, SWII, SWIII	3856	2156	1700
FWI, FWII, FWIII	1172	534	638
All SW, All FW	432	202	230

3.5.1 Overrepresented gene ontology terms

The overrepresented gene ontology (GO) terms for the DE genes common to all six withering time points were investigated. Of the 432 common DE genes, 202 were up-regulated and 230 were down-regulated (**Table 3.5**), regardless of drying intensity (weight loss) or drying rate (fast or slow). The significantly overrepresented GO terms within the root category of biological process are shown in **Figures 3.8** and **3.9,** for up-regulated and down-regulated DE genes respectively. The down-regulated DE genes also corresponded to significantly overrepresented GO terms within the root category of cellular component, and these are represented in **Figure 3.10**. There were 12 overrepresented terms associated with the commonly up-regulated genes, while the down-regulated were associated with 69 (those terms corresponding to $\geq$ 5 percent of DE genes are shown in **Figures 3.9** and **3.10**).

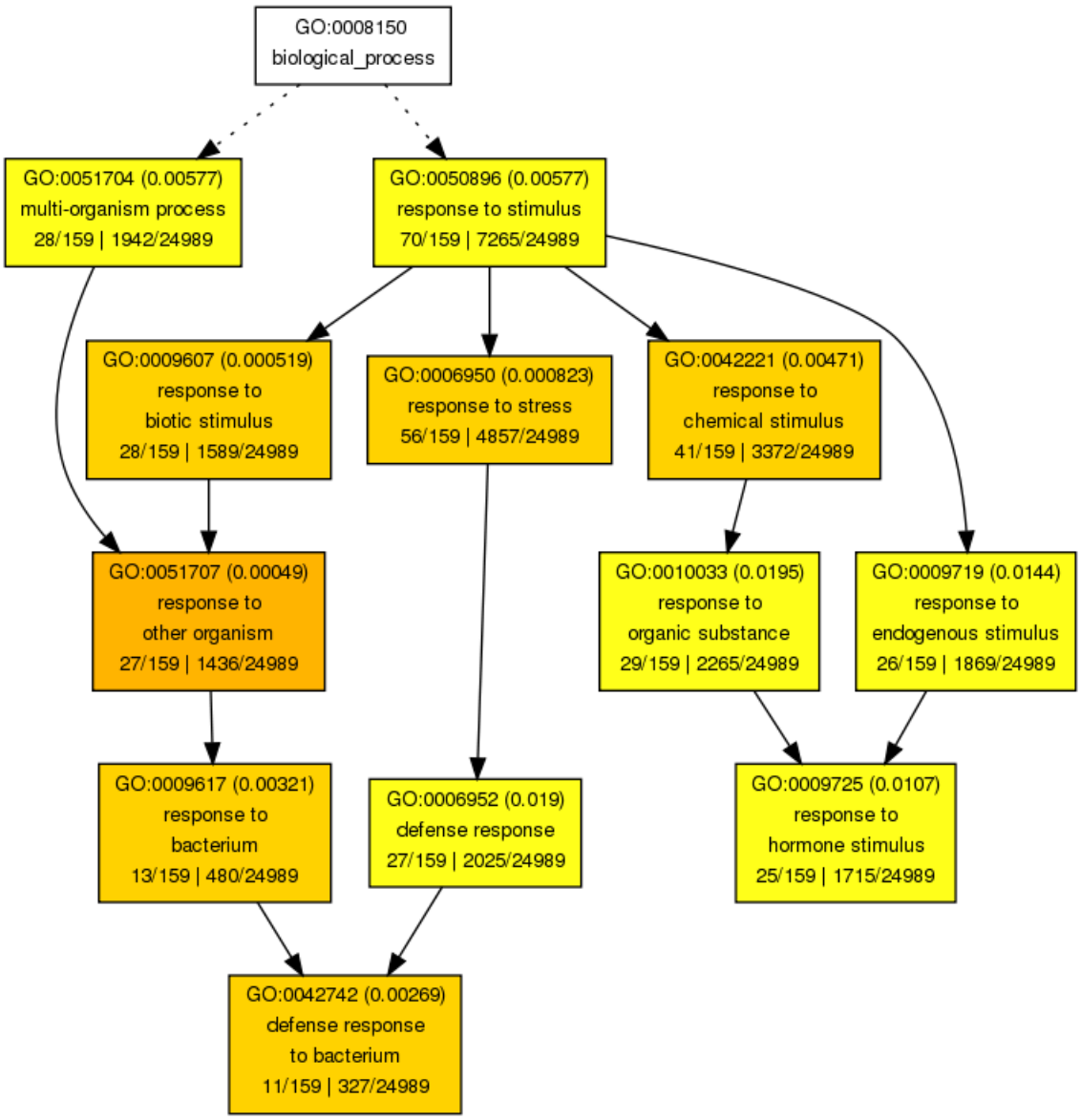


Figure 3.8 Hierarchical tree graph of overrepresented gene ontology (GO) terms in the biological process root category, for up-regulated DE genes common to all postharvest withering time points in *Vitis vinifera* 'Cabernet Franc' berries. The graph is ranked from top to bottom, with each box representing a GO term and arrows demonstrating relationships. Each box contains (from top to bottom and right to left) its GO numerical identifier, adjusted P-value in brackets, term definition, number of corresponding DE genes out of the total analyzed, and number of corresponding genes out of the total in the reference. All terms depicted are significant ($P \leq 0.05$) and the more saturated the colour (from yellow to orange to red), the lower the P-value.

From the 202 up-regulated DE genes, 159 were successfully assigned a GO identifier by using the BLAST method, in combination with the *Vitis vinifera* Genoscope database (see **2.7, Materials and Methods**). Within these 159 GO identifiers, 12 significantly overrepresented GO terms were identified through the use of the web-based toolkit agriGO (Du *et al*., 2010) (**Figure 3.8**). The GO term corresponding to the highest number of up-regulated DE genes was response to stimulus, followed by response to stress. Other than one additional secondary level category, all significant GO terms were associated with response to stimulus and fell within this parent category at a lower rank.

With respect to the down-regulated DE genes, 208 of the 230 were successfully assigned a GO identifier. Similar to the up-regulated genes, there were also 12 significantly overrepresented GO terms identified under the biological process root category (**Figure 3.9**). Most notable was the down-regulation of genes corresponding to the GO term of response to stimulus and its associated lower ranked terms relating to response to light. Down-regulated DE genes were also associated with several other GO terms, including flavonoid metabolic process, pigment metabolic process, cellular nitrogen compound metabolic process and photosynthesis. The down-regulated DE genes common to all withering time points also corresponded with 26 overrepresented GO terms under the cellular component root category (**Figure 3.10**). All of the significant GO terms fell under the second level category of cell, and the vast majority were linked in some capacity to the chloroplast thylakoid membrane category. A few significant terms were also associated with the cell wall and membrane categories.

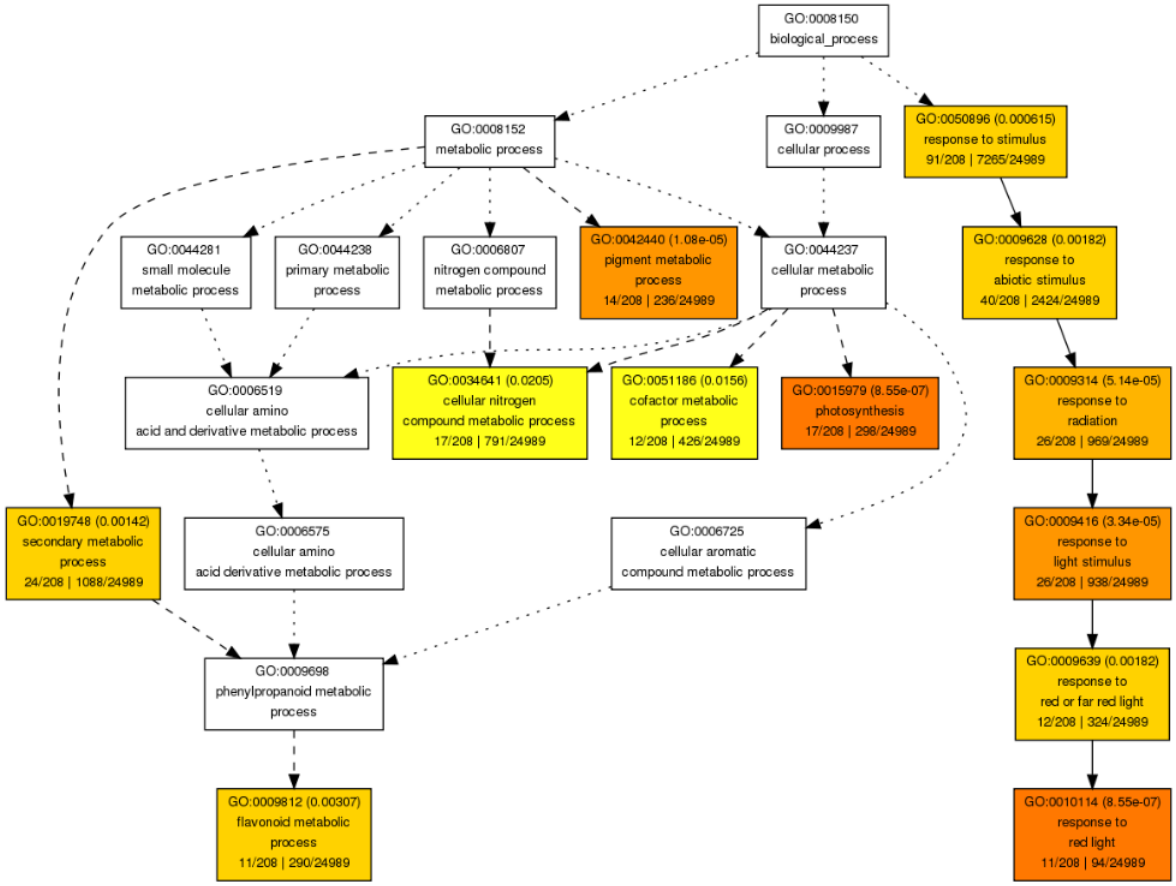


Figure 3.9 Hierarchical tree graph of overrepresented gene ontology (GO) terms in the biological process root category, for down-regulated DE genes common to all postharvest withering time points in *Vitis vinifera* 'Cabernet Franc' berries. The graph is ranked from top to bottom, with each box representing a GO term and arrows demonstrating relationships. Each box contains (from top to bottom and right to left) its GO numerical identifier, adjusted P-value in brackets, term definition, number of corresponding DE genes out of the total analyzed, and number of corresponding genes out of the total in the reference. All terms depicted are significant ($P \leq 0.05$) and the more saturated the colour (from yellow to orange to red), the lower the P-value. Solid, dashed and dotted lines indicate two, one or zero significant term(s) connected by the line, respectively.

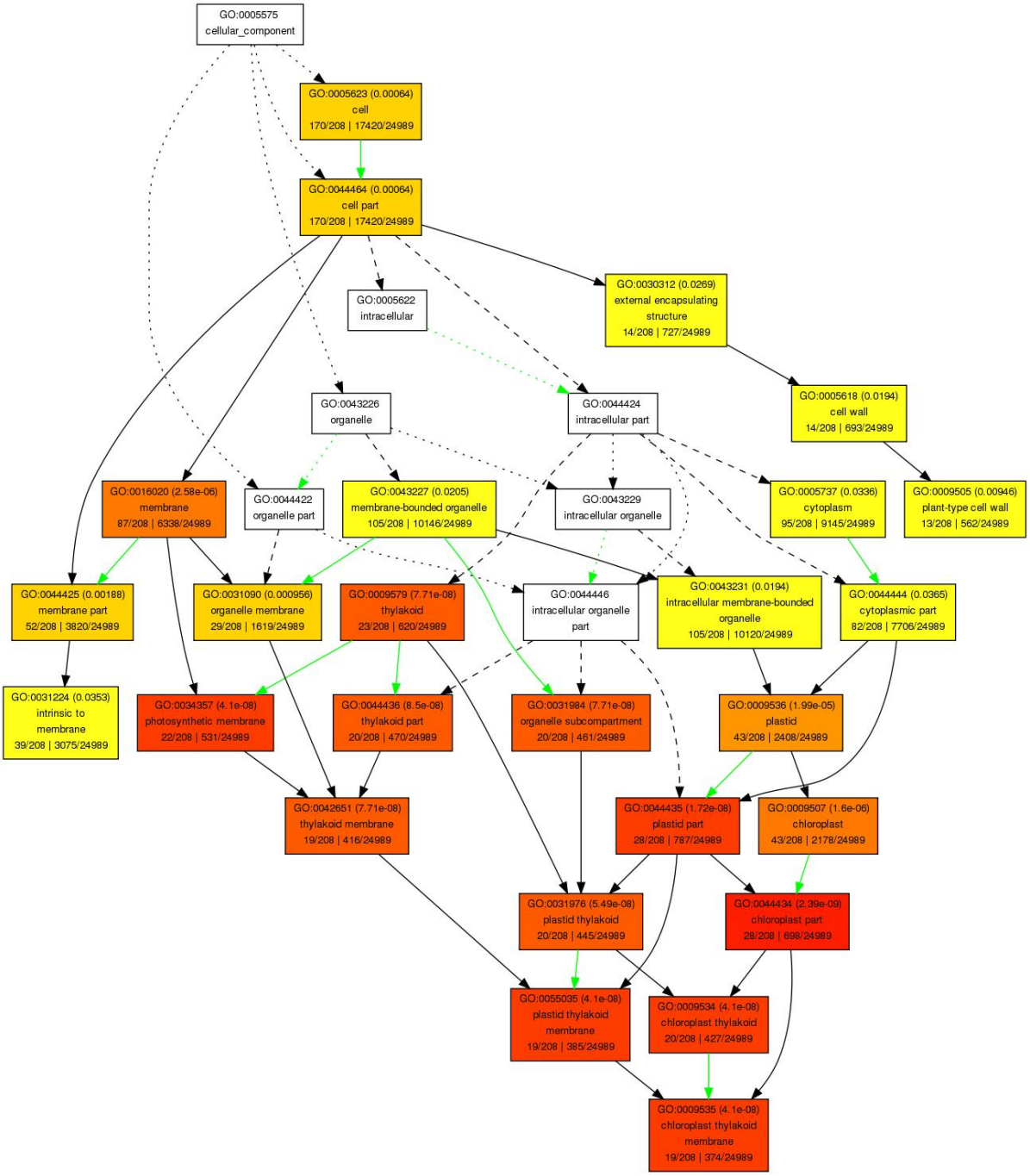


Figure 3.10 Hierarchical tree graph of overrepresented gene ontology (GO) terms in the cellular component root category, for down-regulated DE genes common to all postharvest withering time points in *Vitis vinifera* 'Cabernet Franc' berries. The graph is ranked from top to bottom. Each box represents a GO term and arrows demonstrate relationships. Each box contains (from top to bottom and right to left) its GO numerical identifier, adjusted P-value in brackets, definition, number of corresponding DE genes out of the total analyzed, and number of corresponding genes out of the total in the reference. All terms depicted are significant ($P \leq 0.05$) and the more saturated the colour (from yellow to orange to red), the lower the P-value. Solid, dashed and dotted lines indicate two, one or zero significant term(s) connected by the line, respectively. Green lines indicate negative regulation.

The percent of DE genes associated with each significantly overrepresented GO term was investigated at each withering time point for both slow and fast drying. All those DE genes which were successfully assigned a GO identifier using the Genoscope database were included, as well as all GO terms associated with five percent or greater of the DE genes analyzed. The overrepresented GO terms associated with WI, WII and WIII in slow and fast drying, and the changes over time in the percent DE genes corresponding to each term are shown in **Tables 3.6** and **3.7**. The response to stimulus category and several of its child terms are the most represented across both the slow and fast drying time points, including response to abiotic stimulus, response to biotic stimulus and response to endogenous stimulus. The percent of DE genes associated with the majority of the terms under the response to stimulus parent category remained fairly constant over time as drying intensity increased, and percentages were comparable across both slow and fast dry. The fast drying DE genes were associated with some categories that were not observed in the slow dry, including response to temperature stimulus, response to external stimulus and intracellular signalling cascade. Likewise, there were some categories unique to slow drying DE genes, such as cell, cell part, plasma membrane and chloroplast, which were also associated with the greatest percentage of genes at SWII and SWIII.

The distribution of DE genes within several of the categories was affected both by drying intensity (weight loss) as well as by duration (fast or slow), including secondary metabolic process, cellular aromatic compound metabolic process and transcription factor activity (**Tables 3.6** and **3.7**). The secondary metabolic process and cellular aromatic compound metabolic process categories were only associated with DE genes at the

Table 3.6 Overrepresented gene ontology (GO) term descriptions for differentially expressed (DE) genes at slow postharvest withering time points, showing the associated percent DE genes (≥ 5 percent) in *Vitis vinifera* 'Cabernet Franc' berries. GO term descriptions are indented as rank decreases, with each lower ranked child category under the parent. Percentages and GO Identifiers are only presented where terms are significant. GO terms and the percent associated DE genes are each evaluated separately and genes can be associated with more than one category depending on their function and classification.

GO term description	GO Identifier	Percent DE genes		
		SWI	SWII	SWIII
Biological process				
Developmental process		-	-	-
Post-embryonic development	GO:0009791	9.2	9.2	9.5
Multicellular organismal process	GO:0032501	-	-	20.1
Multi-organism process	GO:0051704	10.2	10.1	10.1
Response to other organism	GO:0051707	8.6	8.4	8.4
Cellular process		-	-	-
Cellular response to stimulus	GO:0051716	-	8.6	-
Cellular response to chemical stimulus	GO:0070887	-	5.0	-
Metabolic process		-	-	-
Secondary metabolic process	GO:0019748	5.7	-	-
Nitrogen compound metabolic process		-	-	-
Cellular nitrogen compound metabolic process	GO:0034641	-	-	5.0
Cellular metabolic process		-	-	-
Cellular aromatic compound metabolic process	GO:0006725	5.6	-	-
Response to stimulus	GO:0050896	34.5	34.6	34.6
Response to endogenous stimulus	GO:0050896	9.9	9.6	9.2
Response to hormone stimulus	GO:0009725	8.9	8.7	8.4
Response to abiotic stimulus	GO:0009628	12.2	12.4	12.5
Response to radiation	GO:0009314	5.4	5.6	5.5
Response to light stimulus	GO:0009416	5.4	5.5	5.4
Response to biotic stimulus	GO:0009607	9.2	9.0	9.1
Response to stress	GO:0006950	22.8	22.4	22.6
Response to chemical stimulus	GO:0042221	16.8	17.0	16.6
Response to organic substance	GO:0010033	-	11.7	11.3
Molecular Function				
Transcription regulator activity		-	-	-
Transcription factor activity	GO:0003700	-	7.1	-
Cellular component				
Cell	GO:0005623	-	72	72.6
Cell part	GO:0044464	-	72	72.6
Membrane		-	-	-
Plasma Membrane	GO:0005886	8.8	8.8	8.7
Intracellular part		-	-	-
Plastid	GO:0009536	11.8	11.3	11.6
Chloroplast	GO:0009507	10.5	10.2	10.4

Table 3.7 Overrepresented gene ontology (GO) term descriptions for differentially expressed (DE) genes at fast postharvest withering time points, showing the associated percent DE genes (≥ 5 percent) in *Vitis vinifera* 'Cabernet Franc' berries. GO term descriptions are indented as rank decreases, with each lower ranked child category under the parent category. Percentages and GO identifiers are included where terms are significant. GO terms and the percent associated DE genes are each evaluated separately and genes can be associated with more than one category depending on their function and classification.

GO term description	GO Identifier	Percent DE genes		
		FWI	FWII	FWIII
Biological process				
Developmental process		-	-	-
Post-embryonic development	GO:0009791	9.0	9.0	8.8
Multi-organism process	GO:0051704	-	-	9.9
Response to other organism	GO:0051707	-	8.3	8.5
Metabolic process		-	-	-
Primary metabolic process		-	-	-
Cellular amino acid derivative metabolic process	GO:0006575	-	5.2	-
Secondary metabolic process	GO:0019748	-	6.7	6.4
Cellular metabolic process		-	-	-
Cellular aromatic compound metabolic process	GO:0006725	-	6.2	6.2
Nitrogen compound metabolic process		-	-	-
Cellular nitrogen compound metabolic process	GO:0034641	-	-	5.2
Signaling		-	-	-
Intracellular signaling cascade	GO:0007242	8.1	-	-
Response to stimulus	GO:0050896	37.3	37.7	37
Response to endogenous stimulus	GO:0050896	11.5	10.9	10.8
Response to hormone stimulus	GO:0009725	10.6	9.9	9.8
Response to abiotic stimulus	GO:0009628	14.9	14.1	13.8
Response to temperature stimulus	GO:0009266	6.3	5.5	5.3
Response to radiation	GO:0009314	6.1	6.0	5.6
Response to light stimulus	GO:0009416	6.1	5.9	5.6
Response to biotic stimulus	GO:0009607	18.9	9.4	9.7
Response to external stimulus	GO:0009605	6.1	5.5	5.5
Response to stress	GO:0006950	-	23.8	23.0
Response to chemical stimulus	GO:0042221	18.9	18.6	17.9
Response to organic substance	GO:0010033	13.1	12.6	12.5
Molecular Function				
Transcription regulator activity		-	-	-
Transcription factor activity	GO:0003700	-	7.8	7.8
Cellular Component				
Cell		-	-	-
Cell part		-	-	-
Plastid	GO:0009536	-	-	11.7
Plastid part	GO:0044435	-	-	5.0

beginning of the process (SWI) in slow drying, and towards the end of the process (FWII and FWIII) in fast drying.

There was a transient association of slow drying DE genes with the transcription factor activity category at SWII. In FWII this association also occurred, however the percentage remained constant into FWIII. Overall, there were a number of common categories which were constantly represented over time, as well as some similar trends between fast and slow withering. However, all of the time points, both within and between drying treatments, have their own distinct list of associated GO term categories and a unique distribution pattern of DE genes (**Tables 3.6** and **3.7**).

An investigation into those overrepresented GO terms associated with the 2,156 up-regulated DE genes common to slow withering (SWI, SWII and SWIII), as well as the 534 common to fast withering (FWI, FWII and FWIII) was performed. Of those DE genes common to the slow withering process time points, 1,724 were assigned GO identifiers and 49 terms were considered significantly overrepresented. With respect to the fast withering time points, of the common DE genes, 428 were assigned GO identifiers and 12 overrepresented terms were considered significant. Lists of significantly overrepresented terms and corresponding percent of associated DE genes for both slow and fast withering are presented in **Tables 3.8** and **3.9**, respectively. The overrepresented GO terms for all slow drying time points corresponded to a much more diverse set of metabolic processes and functional categories (**Table 3.8**), however the fast dry GO terms were almost entirely comprised of abiotic and biotic stress response categories (**Table 3.9**).

Table 3.8 Overrepresented gene ontology (GO) term descriptions, identifiers and P-values for differentially expressed (DE) genes commonly up-regulated at all slow postharvest withering time points, showing the associated percent DE genes in *Vitis vinifera* 'Cabernet Franc' berries. GO terms and the percent associated DE genes are each evaluated separately and genes can be associated with more than one category depending on their function and classification.

GO term description	GO Identifier	Associated DE genes (%)	P-value
response to stimulus	GO:0050896	36.3	3.10E-10
response to stress	GO:0006950	25.2	8.00E-09
response to chemical stimulus	GO:0042221	17.6	2.30E-06
response to organic substance	GO:0010033	12.7	8.80E-07
defense response	GO:0006952	12.2	1.30E-08
multi-organism process	GO:0051704	11.9	5.70E-09
response to endogenous stimulus	GO:0009719	10.9	5.60E-07
response to biotic stimulus	GO:0009607	10.8	1.20E-11
protein amino acid phosphorylation	GO:0006468	10.6	0.00027
protein serine/threonine kinase activity	GO:0004674	10.2	2.50E-06
response to other organism	GO:0051707	10.0	2.50E-11
plasma membrane	GO:0005886	9.7	3.70E-05
response to hormone stimulus	GO:0009725	9.7	1.40E-05
protein tyrosine kinase activity	GO:0004713	9.2	2.60E-07
post-embryonic development	GO:0009791	8.4	0.00026
transcription factor activity	GO:0003700	8.0	3.80E-05
cellular aromatic compound metabolic process	GO:0006725	6.7	2.00E-07
immune system process	GO:0002376	5.7	6.70E-05
cellular amino acid derivative metabolic process	GO:0006575	5.6	1.10E-07
immune response	GO:0006955	5.4	4.30E-05
innate immune response	GO:0045087	4.8	8.40E-05
cellular nitrogen compound metabolic process	GO:0034641	4.8	0.00027
phenylpropanoid metabolic process	GO:0009698	4.5	4.70E-07
aromatic compound biosynthetic process	GO:0019438	4.3	1.10E-06
cellular amino acid derivative biosynthetic process	GO:0042398	4.2	1.50E-06
response to inorganic substance	GO:0010035	3.7	4.70E-12
response to bacterium	GO:0009617	3.6	8.90E-06
phenylpropanoid biosynthetic process	GO:0009699	3.5	1.30E-05
response to fungus	GO:0009620	3.4	1.70E-06
response to jasmonic acid stimulus	GO:0009753	3.2	1.20E-05
regulation of response to stimulus	GO:0048583	3.0	3.10E-05
defense response to bacterium	GO:0042742	2.6	7.40E-05
response to heat	GO:0009408	2.5	6.60E-05
response to wounding	GO:0009611	2.4	9.90E-05
response to metal ion	GO:0010038	2.1	0.00017
regulation of defense response	GO:0031347	2.0	2.70E-06
response to reactive oxygen species	GO:0000302	2.0	1.90E-05
host programmed cell death induced by symbiont	GO:0034050	2.0	2.30E-05
plant-type hypersensitive response	GO:0009626	2.0	2.30E-05
regulation of response to stress	GO:0080134	2.0	4.80E-05
response to hydrogen peroxide	GO:0042542	1.9	1.40E-06
response to light intensity	GO:0009642	1.7	1.40E-05
multicellular organism reproduction	GO:0032504	1.6	0.00014
regulation of immune response	GO:0050776	1.3	0.00016
trihydroxystilbene synthase activity	GO:0050350	1.2	5.80E-09
reproductive cellular process	GO:0048610	1.1	1.30E-09
lipid localization	GO:0010876	0.8	1.30E-08
response to 1-aminocyclopropane-1-carboxylic acid	GO:0009961	0.5	7.90E-05
microgametogenesis	GO:0055046	0.3	0.00022

Table 3.9 Overrepresented gene ontology (GO) term descriptions, identifiers and P-values for differentially expressed (DE) genes commonly up-regulated at all fast postharvest withering time points, showing the associated percent DE genes in *Vitis vinifera* 'Cabernet Franc' berries. GO terms and the percent associated DE genes are each evaluated separately and genes can be associated with more than one category depending on their function and classification.

GO term description	GO Identifier	Associated DE genes (%)	P-value (%)
response to stimulus	GO:0050896	42.4	9.80E-09
response to stress	GO:0006950	29.2	1.70E-06
response to chemical stimulus	GO:0042221	22.4	8.00E-07
response to organic substance	GO:0010033	16.0	5.60E-06
response to endogenous stimulus	GO:0009719	14.4	1.50E-06
response to hormone stimulus	GO:0009725	13.2	4.10E-06
response to abscisic acid stimulus	GO:0009737	6.8	9.80E-06
response to inorganic substance	GO:0010035	4.9	3.50E-07
response to wounding	GO:0009611	4.5	3.80E-06
response to metal ion	GO:0010038	3.3	0.00023
second-messenger-mediated signaling	GO:0019932	2.1	5.80E-05
calcium-mediated signaling	GO:0019722	1.4	0.00017

3.6 Expression patterns associated with several genes of interest

The expression patterns associated with selection of several genes of interest were investigated across both fast and slow withering. These genes are known to be involved in abiotic stress responses in plants, and/or their behaviour has been investigated in previous postharvest *appassimento* studies conducted in Italy, using Northern blot, qPCR, microarray and AFLP-TP analyses.

3.6.1 CBF transcription factors

The *Vitis vinifera* reference genome (NCBI *Vitis vinifera* Annotation Release 101) contained two *CBF* genes, *CBF3* and *CBF4*, and their general expression patterns across all three withering time points for both fast and slow drying treatments were investigated. Changes in expression for *CBF3* and *CBF4* were found only in slow drying and only in fast drying, respectively (**Figure 3.11**). There was an increase in *CBF3* transcripts at SWI, followed by a return to harvest levels at SWII and a subsequent increase at SWIII (**Figure 3.11A**). For *CBF4*, there was a drop in transcript levels associated with FWII and a return to harvest levels by FWIII (**Figure 3.11B**).

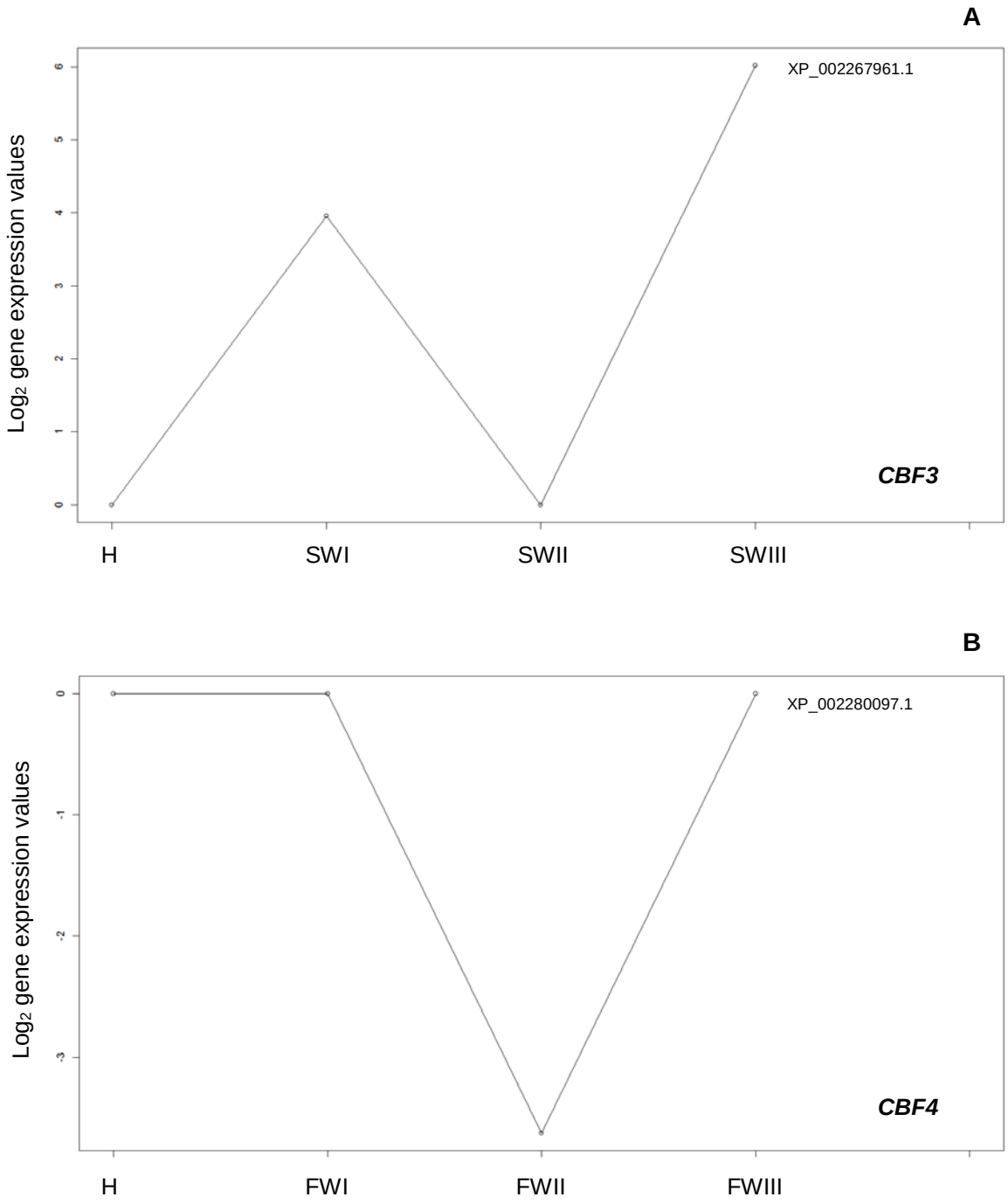


Figure 3.11 Expression profiles of *CBF* genes (log_2 values) over time during postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries. (**A**) Gene expression of *CBF3* (XP_002267961.1) at each sampling time point during slow withering. (**B**) Gene expression of *CBF4* (XP_002280097.1) at each sampling time point during fast withering.

3.6.2 WRKY transcription factors

The *Vitis vinifera* reference genome contained 61 different genes for WRKY transcription factors (TFs), which included those considered probable. Of these genes, 34 showed differential expression during the course of slow withering, and 16 during fast withering (**Figures 3.12** and **3.13**). With respect to slow drying, 30 of the 34 genes showing differential expression were up-regulated at one or more of the withering time points (**Figure 3.12**). A smaller amount of *WRKY* genes were differentially expressed in fast withering; however, similar to slow withering the majority (11 of the 16 genes) were up-regulated at one or more of the time points (**Figure 3.13**). All 11 of the up-regulated *WRKY* genes during fast withering were included among those 30 genes up-regulated during slow withering. Of the five down-regulated *WRKY* genes during fast withering, one was up-regulated during slow drying (XP_002267867.3), one was down-regulated (XP_002270750.3) and three were not differentially expressed at any of the slow withering time points.

3.6.3 MYB transcription factors

The *Vitis vinifera* reference genome contained 134 different genes for MYB transcription factors, which included those considered probable. The slow withering time points had a higher number of DE genes as compared to fast, with 45 and 27 *MYB* genes showing changes in expression respectively. Overall, 68.9 percent of the DE genes were up-regulated at one or more time points during slow withering and 63 percent were up-regulated during fast withering (**Figure S1**).

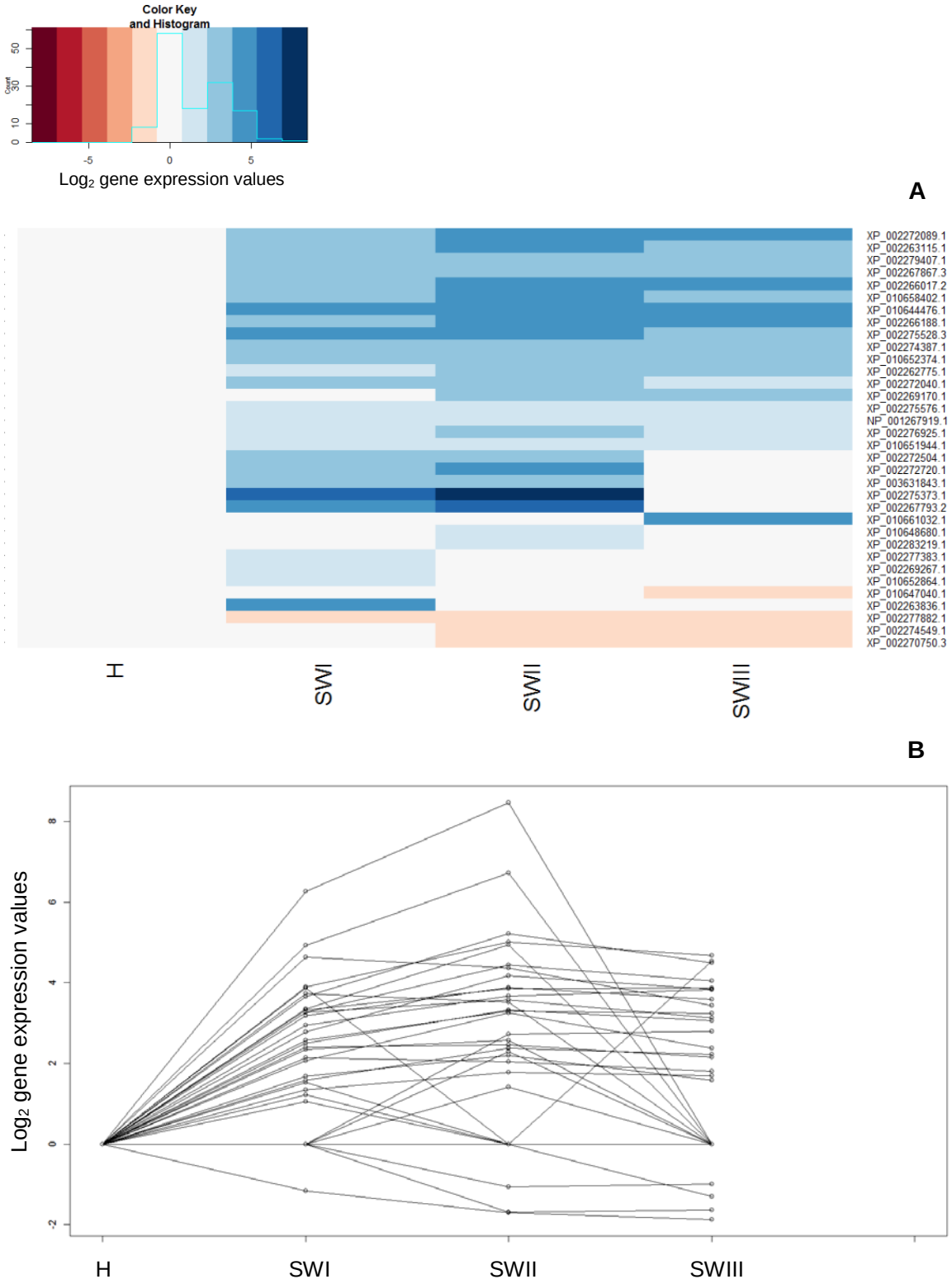


Figure 3.12 Expression profiles for *WRKY* genes (log_2 values) over time during slow postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries. (**A**) Heat map and (**B**) line graph demonstrating changes in expression values at each sampling time point.

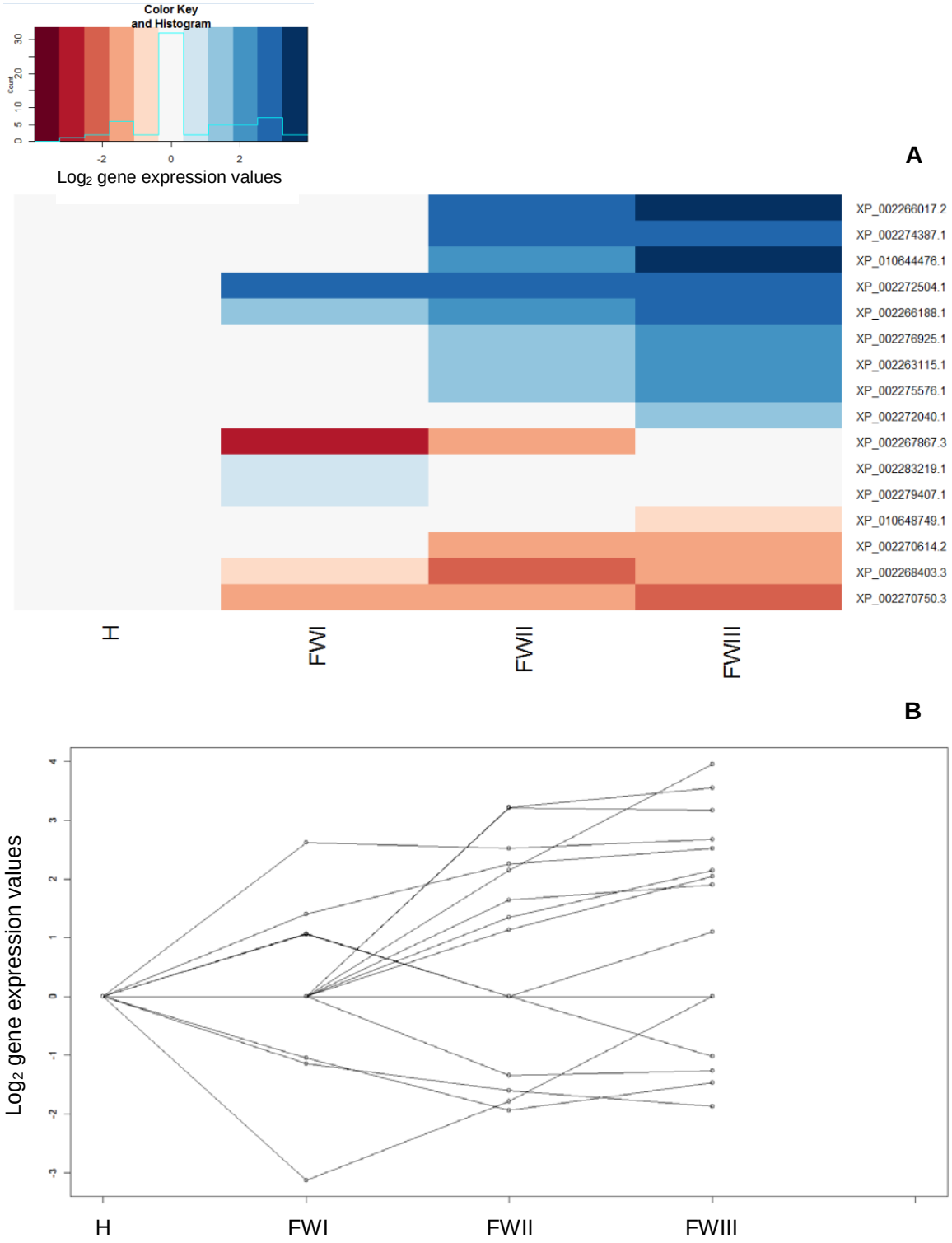


Figure 3.13 Expression profiles for *WRKY* genes (log_2 values) over time during fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries. (**A**) Heat map and (**B**) line graph demonstrating changes in expression values at each sampling time point.

Of the 134 MYB genes in the reference genome, two of these corresponded to MYBA transcription factors (NP_001268002.1 and XP_002274992.2). There were no changes in expression, as compared to harvest transcript levels, for either of these *MYBA* genes at any of the time points during fast or slow withering.

3.6.4 Phenylalanine ammonia lyase

The *Vitis vinifera* reference genome contained 16 different genes coding for phenylalanine ammonia lyase (PAL). Of these, 14 were differentially expressed during slow drying, demonstrating a strong up-regulation of all but one gene at one or more of the time points (**Figure 3.14A**). There were six *PAL* genes differentially expressed during fast drying, of which half were up-regulated and half were down-regulated at one or more time points (**Figure 3.14B**).

3.6.5 Chalcone synthase

There were eight different genes coding for chalcone synthase (CHS) in the *Vitis vinifera* reference genome. Only one of these genes was differentially expressed during fast withering (NP_001267879.1). This gene was down-regulated by FWI and remained as such through FWIII (**Figure 3.15B**). Across the slow drying time points, five *CHS* genes were differentially expressed, with three down-regulated and two up-regulated (**Figure 3.15A**). The down-regulated gene observed during fast withering was also among those down-regulated across the slow withering time points.

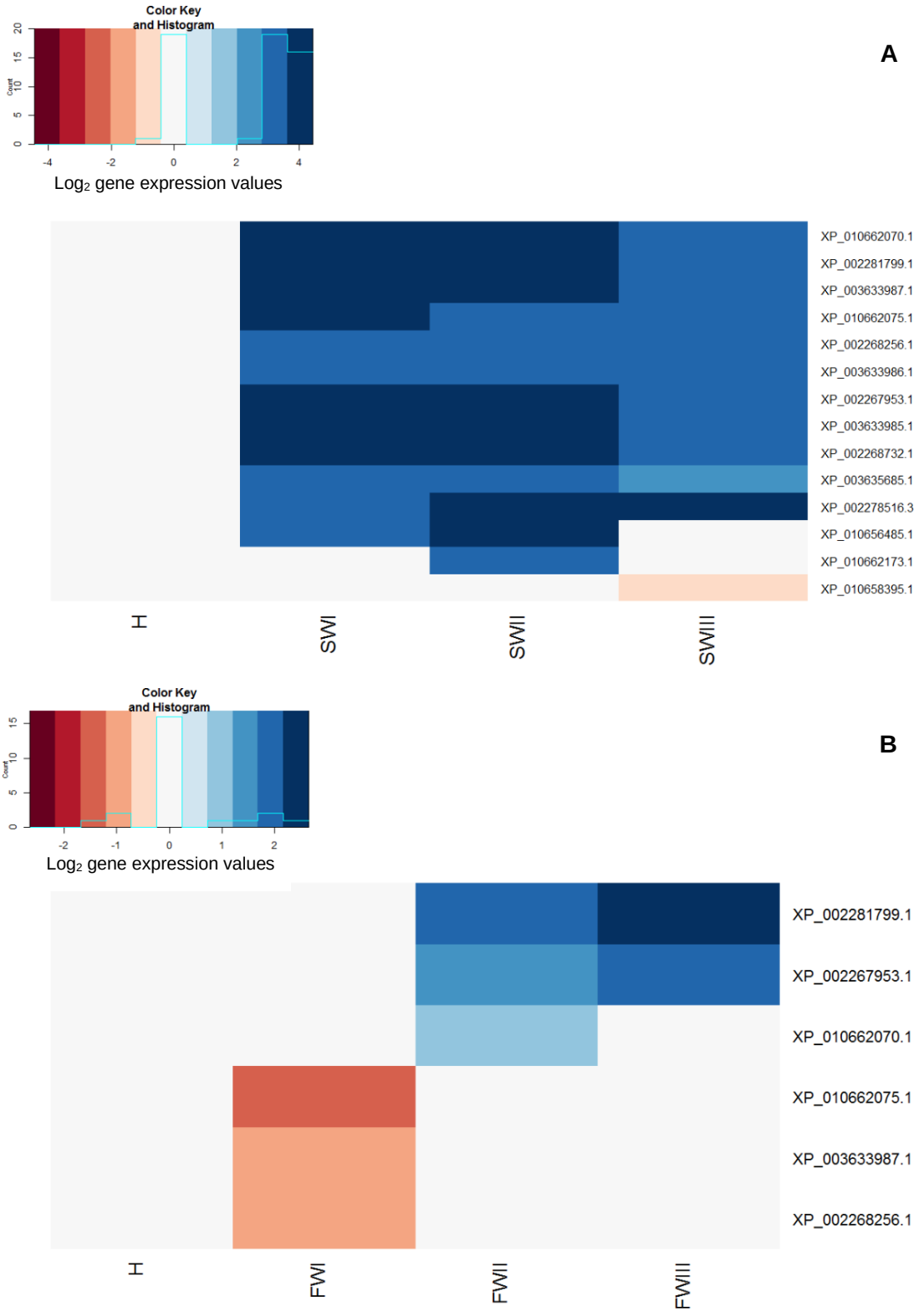


Figure 3.14 Heat maps demonstrating expression profiles for differentially expressed phenylalanine ammonia lyase genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

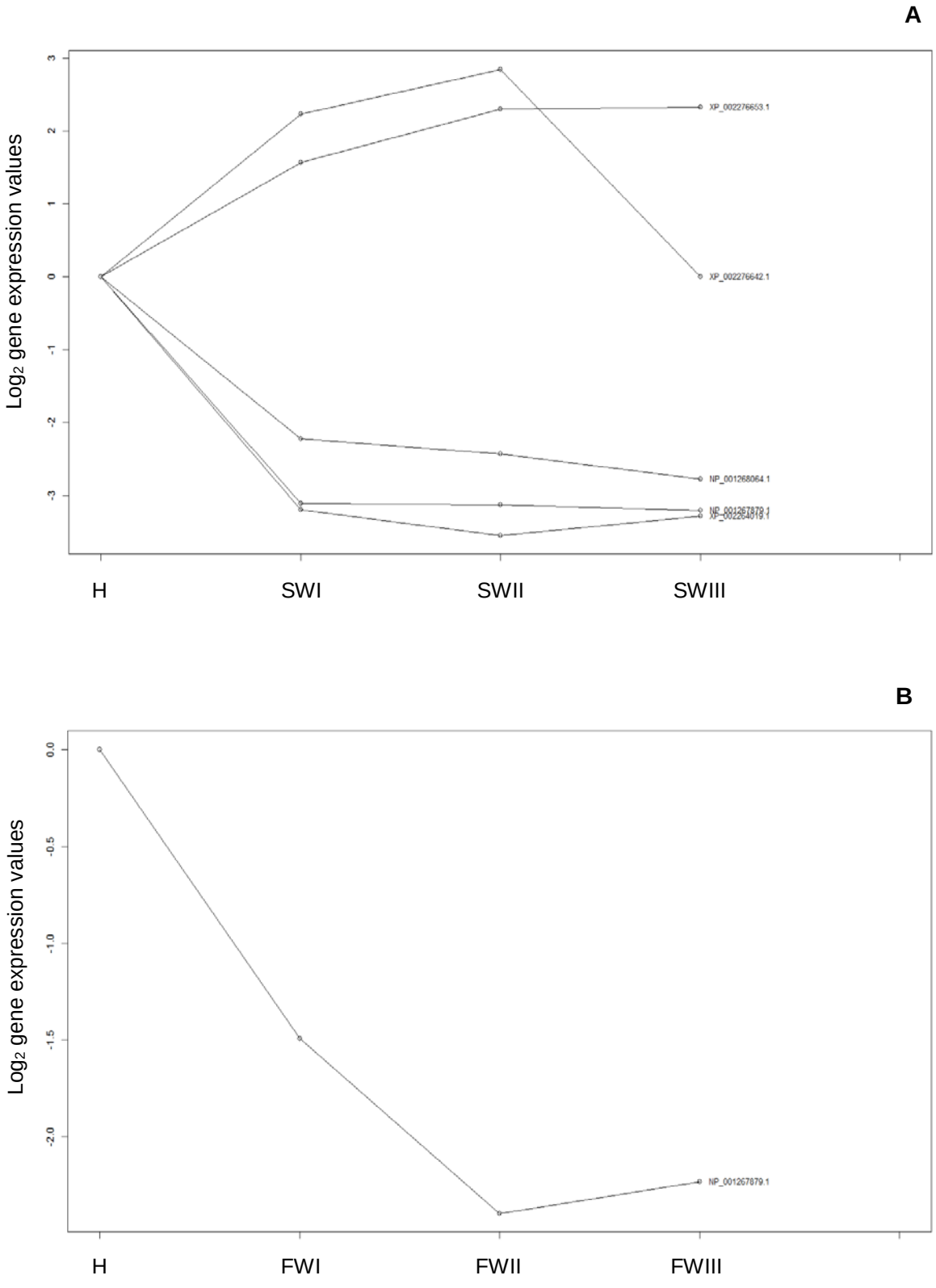


Figure 3.15 Line graphs demonstrating expression profiles for differentially expressed chalcone synthase genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

3.6.6 UDP-glucose:flavonoid 3-O-glucosyltransferase

There was one gene in the *Vitis vinifera* reference genome coding for UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) (XP_010657882.1). This gene was not differentially expressed at any of the slow withering time points and was down-regulated transiently at WII in the fast withering treatment (**Figure S2**).

3.6.7 Flavonol synthase

There were nine different genes coding for flavonol synthase (FLS) in the *Vitis vinifera* reference genome. Of the six different genes that showed differential expression across slow withering, half of them were up-regulated and half were down-regulated at one or more time points (**Figure 3.16A**). Similar results were observed across the fast withering time points (**Figure 3.16B**), with up-regulation of one gene and down-regulation of two genes. The two down-regulated genes were among those also down-regulated during slow withering (XP_002278024.1 and XP_002285839.1).

3.6.8 Stilbene synthase

The *Vitis vinifera* reference genome contained 37 different genes coding for stilbene synthase (STS). There was a strong up-regulation of 35 of these genes by WI in slow withering (**Figure 3.17**). All but three of these followed a similar expression pattern and were still up-regulated at WIII. Only one *STS* gene was down-regulated in slow withering. There was an up-regulation of 25 *STS* genes in the fast withering, however the fold change values were not as high as observed in slow withering, and up-regulation most often occurred by the WII time point (**Figure 3.18**). There were also four *STS* genes which were transiently down-regulated together at WI in the fast withering treatment.

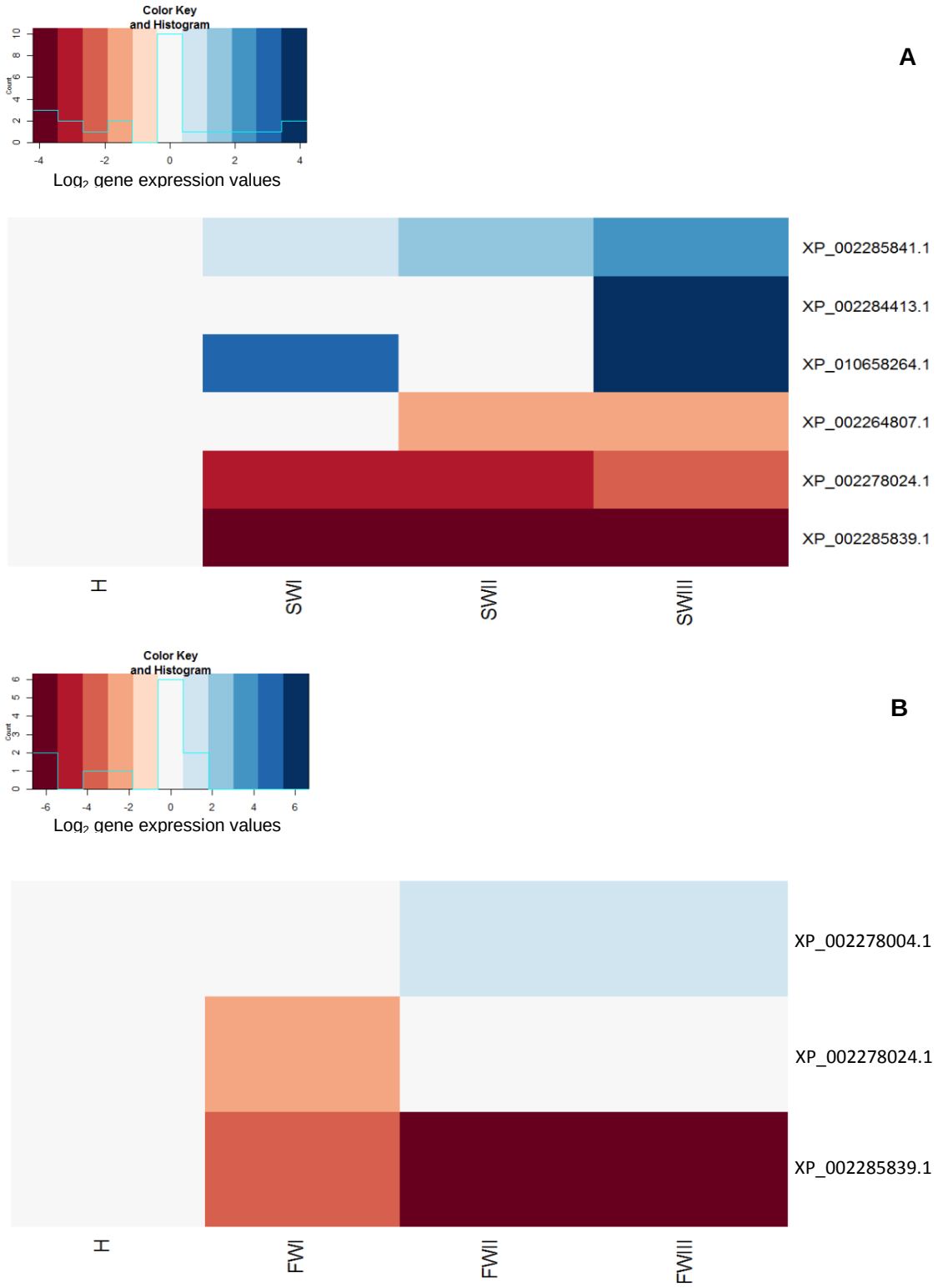


Figure 3.16 Heat maps demonstrating expression profiles for differentially expressed flavonol synthase genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

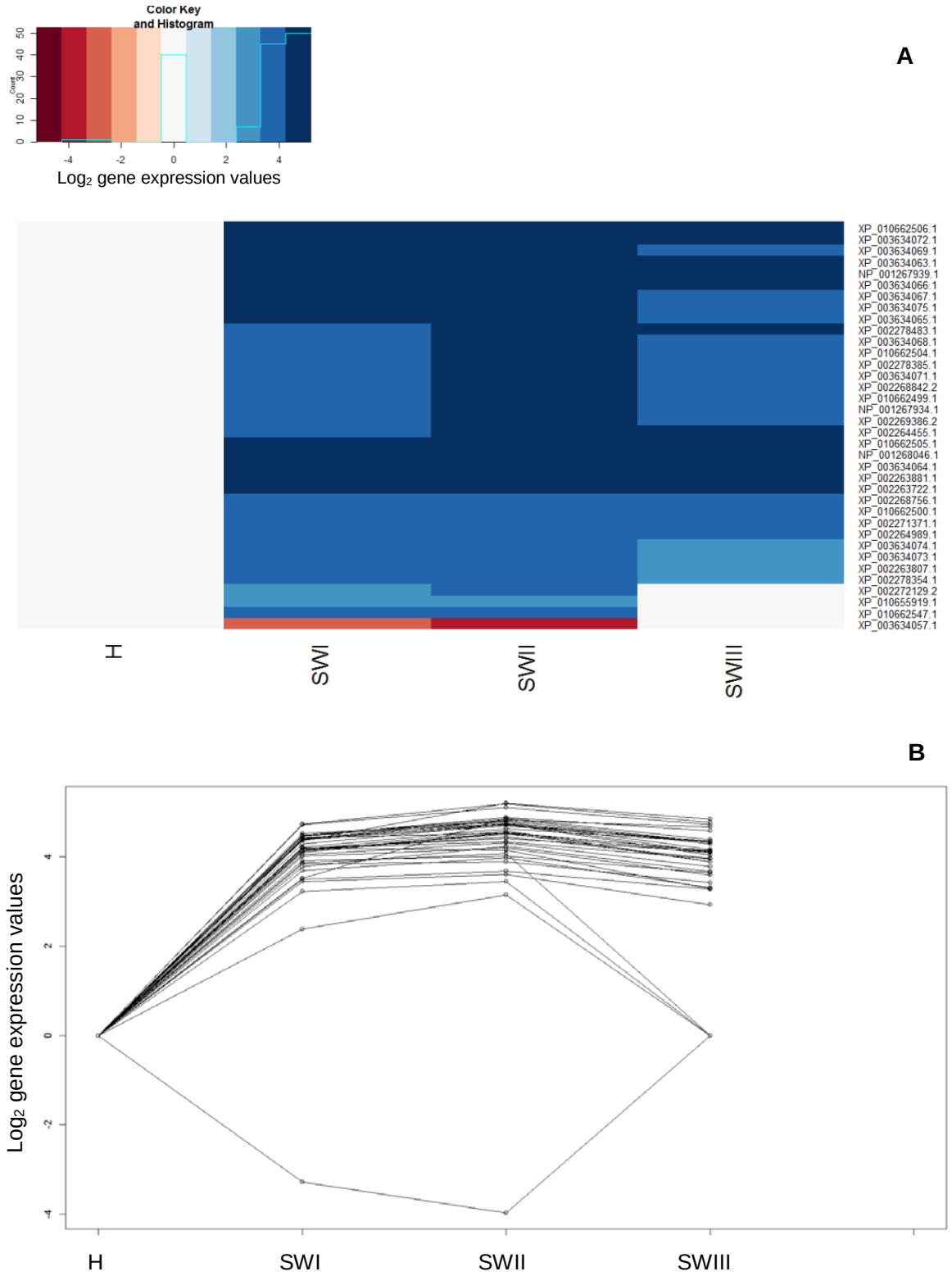


Figure 3.17 Expression profiles for stilbene synthase genes (log_2 values) over time during slow postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries. (**A**) Heat map and (**B**) line graph demonstrating changes in expression values at each sampling time point.

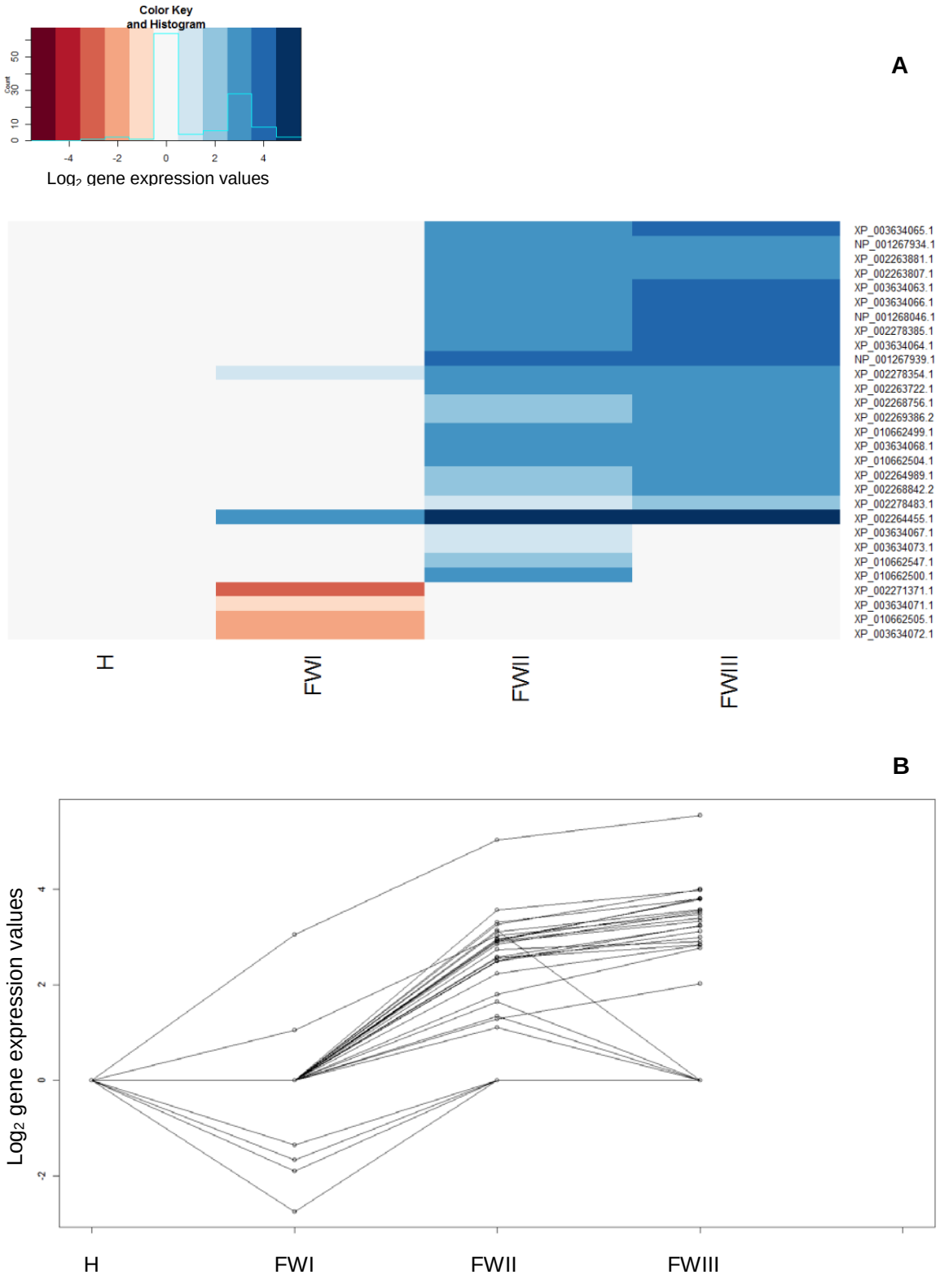


Figure 3.18 Expression profiles for stilbene synthase genes (log_2 values) over time during fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries. (**A**) Heat map and (**B**) line graph demonstrating changes in expression values at each sampling time point.

3.6.9 Dehydrins

There were two genes coding for dehydrins in the *Vitis vinifera* reference genome. Only one of these genes (NP_001268221.1) was differentially expressed across the slow withering time points, while fast withering did not show any changes in dehydrin gene expression. In slow withering, the differentially expressed dehydrin gene was up-regulated by WI and remained up-regulated at WII and WIII time points (**Figure S3**).

3.6.10 Alcohol dehydrogenase

The *Vitis vinifera* reference genome contained 16 different genes coding for alcohol dehydrogenase (ADH). One half of these showed differential expression across slow withering, and of those eight genes, one half were up-regulated and the other half down-regulated at one or more time points (**Figure 3.19A**). Only two *ADH* genes were differentially expressed across fast withering, and these were also split with one up-regulated and one down-regulated (**Figure 3.19B**). The sole alcohol dehydrogenase 2 gene from the reference genome (NP_001268083.1) was down-regulated at all three slow withering time points, and showed no differential expression across the fast dry samples. There were three alcohol dehydrogenase 7 genes in the reference genome, and two of these were up-regulated in slow withering. The third alcohol dehydrogenase 7 gene was down-regulated by WII in both slow and fast withering (NP_001268090.1).

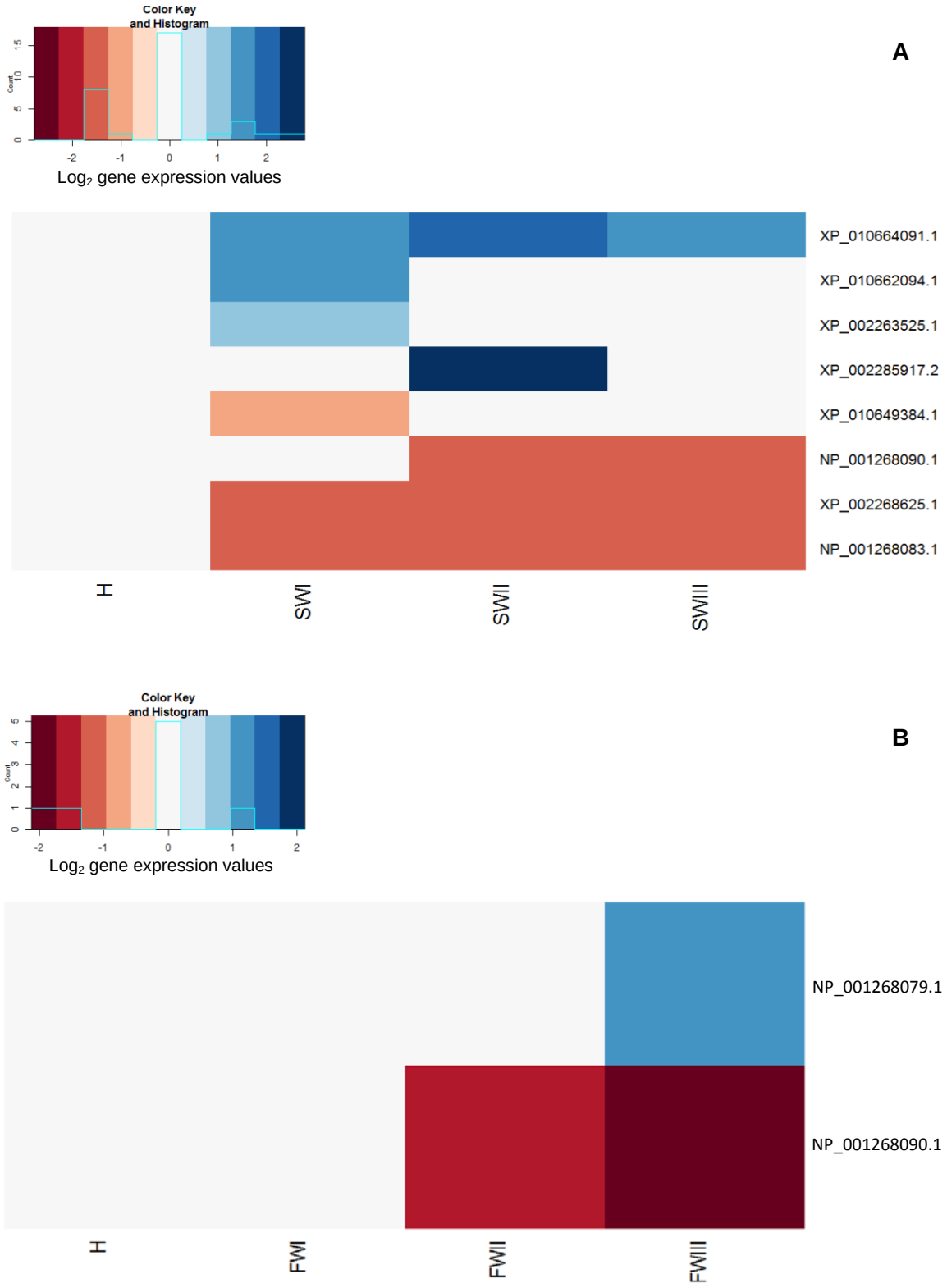


Figure 3.19 Heat maps demonstrating expression profiles for differentially expressed alcohol dehydrogenase genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

3.6.11 Trehalose-phosphate phosphatase

The *Vitis vinifera* reference genome contained seven different genes coding for trehalose-phosphate phosphatase (TPP), which included those considered probable. Five genes showed differential regulation across slow withering and four of these were up-regulated (**Figure 3.20A**). Three of the up-regulated genes were increased at every time point, and the remaining gene was up-regulated only at the end of the process at WIII. There were three genes differentially expressed across fast withering and they were all up-regulated at every time point (**Figure 3.20B**). Two of these DE fast withering genes (XP_003631447.1 and XP_002264471.2) were also among the four up-regulated across slow withering.

3.6.12 Genes exhibiting similar regulation in both slow and fast withering

Several genes of interest were investigated to search for those which may have exhibited similar regulation profiles across both slow and fast withering. As mentioned previously, these genes were considered to be of interest because they are known to be involved in abiotic stress responses in plants, and/or their behaviour has been investigated in previous postharvest *appassimento* studies conducted in Italy. All genes contained in the *Vitis vinifera* reference genome corresponding to the following were examined: 1-aminocyclopropane-1-carboxylate oxidase, *S*-adenosyl methionine synthase, CBF transcription factor, WRKY transcription factor, MYB transcription factor, phenylalanine ammonia lyase, chalcone synthase, flavonol synthase, UDP-glucose:flavonoid 3-O-glucosyltransferase, stilbene synthase, dehydrin, alcohol dehydrogenase and trehalose-phosphate phosphatase. From these genes, 11 exhibited the same general expression

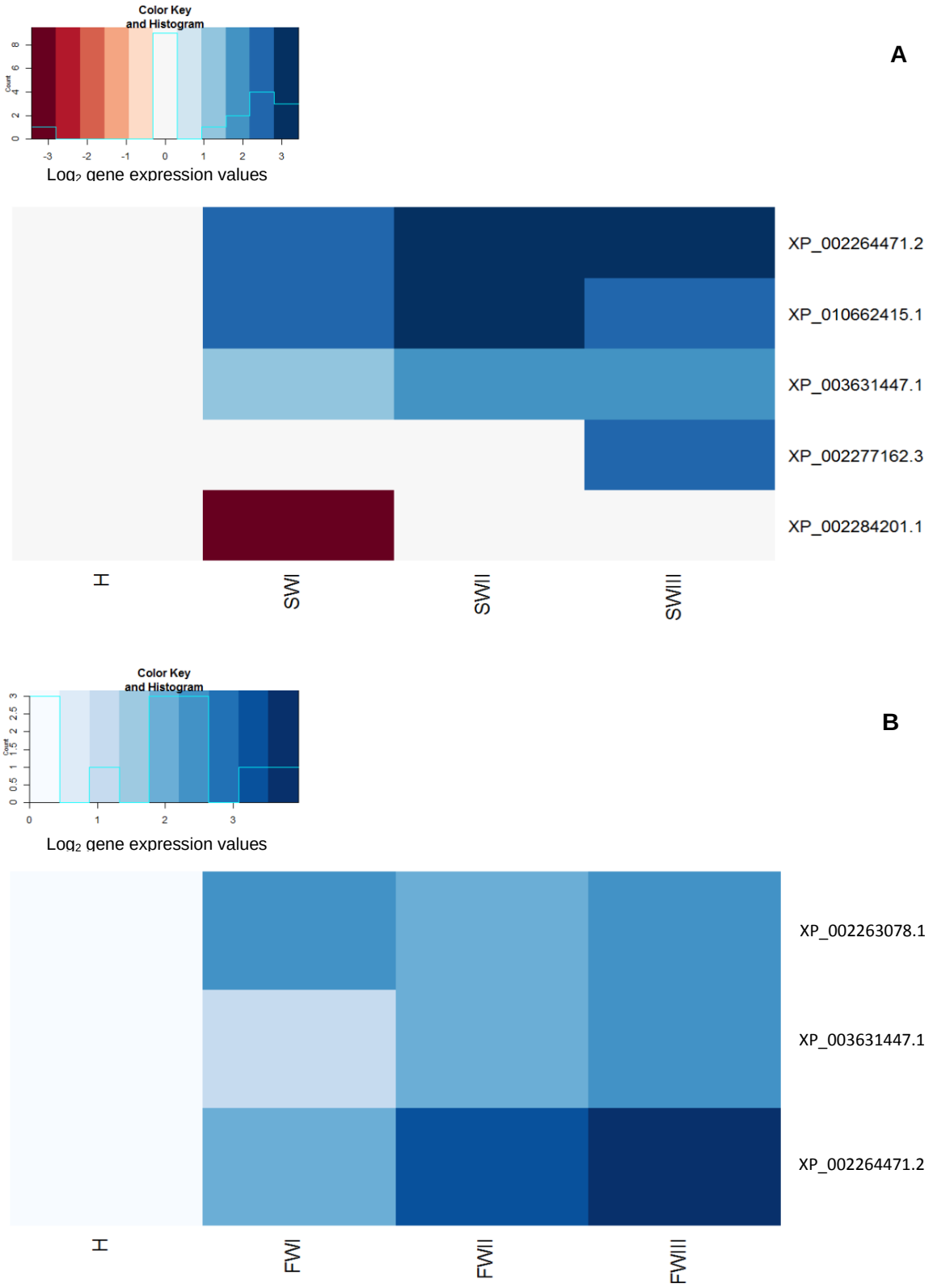


Figure 3.20 Heat maps demonstrating expression profiles for differentially expressed trehalose-phosphate phosphatase genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

profiles (up-regulated, down-regulated or no change from harvest transcript level), across WI, WII and WIII in both slow and fast withering. These genes are presented in **Table 3.10** and represent those which behave in the same general manner during drying, independent of the rate and temperature of the withering process.

Table 3.10 Genes exhibiting similar general expression profiles across both slow and fast postharvest withering time points in *Vitis vinifera* 'Cabernet Franc' berries. Changes in expression are indicated as up-regulated (up), down-regulated (down) and no change from harvest transcript level (-).

Gene	Accession[z]	General expression profile			
		H	WI	WII	WIII
WRKY 11 TF (probable)	XP_002266188.1	-	up	up	up
MYB108-like TF	NP_001267991.1	-	up	up	up
MYB-related TF	XP_010661068.1	-	down	down	down
MYB4-related TF	XP_002264563.2	-	up	-	-
Chalcone synthase	NP_001267879.1	-	down	down	down
Flavonol synthase	XP_002285839.1	-	down	down	down
Stilbene synthase 5	XP_002278354.1	-	up	up	up
Stilbene synthase 1	XP_002264455.1	-	up	up	up
Alcohol dehydrogenase 7	NP_001268090.1	-	-	down	down
Trehalose-phosphate phosphatase (probable)	XP_002264471.2	-	up	up	up
Trehalose-phosphate phosphatase (probable)	XP_003631447.1	-	up	up	up

[z] Genoscope accession number (NCBI *Vitis vinifera* Annotation Release 101 genome)

3.7 Detection of fungal transcripts during postharvest withering

Detection of fungal transcript presence during drying was accomplished by determining the alignment rates for the RNA-Seq reads from each time point using the *Botrytis cinerea* B05.10 reference genome (NCBI Genbank) (**2.7, Materials and Methods**). Overall, there were no significant differences found between any of the rates, when considering all time points and both slow and fast withering (**Table 3.11**).

Table 3.11 Percent alignment rates of raw reads to the *Botrytis* reference genome for each *Vitis vinifera* 'Cabernet Franc' berry sample submitted for RNA-Seq. Total RNA was isolated from berry tissue at harvest (H), fast withering (FW) and slow withering (SW) sampling time points.

Sample name	Alignment rate[y] (%)
H	
Rep 1	4.31[z]
Rep 2	2.62
Rep 3	1.96
FWI	
Rep 1	4.36
Rep 2	8.03
Rep 3	8.44
FWII	
Rep 1	0.73
Rep 2	6.86
Rep 3	10.38
FWIII	
Rep 1	2.64
Rep 2	9.21
Rep 3	3.21
SWI	
Rep 1	2.87
Rep 2	9.91
Rep 3	6.21
SWII	
Rep 1	0.42
Rep 2	2.05
Rep 3	0.24
SWIII	
Rep 1	3.15
Rep 2	0.52
Rep 3	5.85

[y]Aligned with Bowtie software; Mapped to NCBI Genbank *Botrytis cinerea B05.10* genome

[z]Values are not significantly different according to ANOVA ($P < 0.05$)

Chapter 4. Discussion

4.1 Withering rate and intensity affect grape berry composition and metabolism

The present study describes the successful use of a fully controlled novel air flow system, to achieve both a fast and a slow *appassimento* withering process in 'Cabernet Franc' grape berries. With respect to slow withering, this method is able to maintain high berry quality and can achieve the requirements of the traditional uncontrolled *appassimento* technique (**Figure 3.1**), such as a duration lasting between 90 to 150 days (Accordini, 2013), and a weight loss of 35 to 40 percent (Paronetto and Dellaglio, 2011; Tonutti *et al*., 2004). The controlled system was also able to dry berries more rapidly, in 20 days, while maintaining high quality berries and achieving a weight loss of over 30 percent (**Figure 3.1**). As expected, drying intensity impacted berry composition, and total soluble solids content (^{o}Brix) increased over time for both fast and slow withering (**Table 3.1**) (Accordini, 2013; Bellincontro *et al*., 2009; Chkaiban *et al*., 2007; Cirilli *et al*., 2012; Costantini *et al*., 2006; Mencarelli *et al*., 2010; Versari *et al*., 2001; Zamboni *et al*., 2008). As was also anticipated, temperature affected the rate of drying (Barbanti *et al*., 2008), with the 25 ^{o}C treatment achieving 29 $\pm$ 1 ^{o}Brix in 20 days (fast withering) and the 6 ^{o}C treatment reaching this target in 132 days (slow withering) (**Table 2.1** and **Figure 3.1**).

Drying intensity also affected titratable acidity (TA) in the berries. Despite the expected concentration of acidity due to water loss, there was a common decrease in TA by the end of drying in both treatments (**Tables 3.1** and **3.2**). A decrease in TA during withering is a common response which has been observed in many grape varieties (Bellincontro *et al*., 2016). TA decreased slightly by the end of slow drying from 7.400 g/L to 7.193 g/L, although this was not significant, and by the end of the fast drying the TA

had dropped to a significantly lower level of 6.750 g/L (**Table 3.1**). The TA pattern observed in fast withering, consisting of an initial increase followed by a decrease (**Table 3.1**), is likely due to an initial rapid weight loss concentration effect, followed by consumption primarily due to malic acid respiration (Bellincontro *et al*., 2004; Centioni *et al*., 2014; Chkaiban *et al*., 2007). It has been demonstrated that berry respiration continues during postharvest withering (Costantini *et al*., 2006; Morozova *et al*., 2016), and malic acid is often consumed as a substrate in grapes during respiration (Sweetman, 2009). Higher respiration rates have been observed in berries withered at 20 ^{o}C as compared to 10 ^{o}C (Bellincontro *et al*., 2009), which may provide an explanation for the lower TA concentration observed in the present study by the end of fast withering, as compared to slow (**Table 3.1**). The observation of an increase in TA during fast postharvest withering, followed by a decrease has also been observed in 'Malvasia' and 'Trebbiano' (Bellincontro *et al*., 2004) grapes in Italy.

In the present study, a decrease in TA at W1 during slow drying occurred corresponding to a 17.6 percent weight loss, which was not observed in the fast. This decrease was followed by an increase in TA at 32.9 percent weight loss (SWII), to levels similar to that observed in FWI, and finally a decrease to a value not significantly different from harvest levels (**Table 3.1**). A decrease in TA at approximately 20 percent cluster weight loss, followed by an increase in TA at approximately 30 percent cluster weight loss, is a pattern that has also been observed in slow withering for 'Sangiovese' (Zoccatelli *et al*., 2013) 'Corvina' and 'Corvinone' grapes (Bellincontro *et al*., 2016). However, patterns of TA during drying appear to be highly dependent on the grape cultivar (Bellincontro *et al*., 2004; Bellincontro *et al*., 2016; Zoccatelli *et al*., 2013). Morozova *et al*. (2016)

demonstrated a decreasing and increasing fluctuation in the respiration of grape berries (measured as heat rate) dried in a partially controlled environment over 120 days. A variable respiration rate, in combination with a concentration effect due to water loss, could provide an explanation for the pattern observed in TA levels during slow drying in the present study.

As discussed, the effect of drying intensity and rate on berry composition is apparent through observing the changes in TA with cluster fresh weight loss (**Table 3.1**). The effect of drying rate on grape berry composition is also revealed by comparing TA levels between fast and slow treatments at similar levels of weight loss or drying intensity (**Table 3.1**). The weight loss percentages at WI for both slow and fast dry were significantly similar to each other, as were the weight loss values at WII. However, the TA values were significantly higher at WI for fast dry as compared to slow, and significantly lower at WII for slow dry as compared to fast (**Table 3.1**). This trend confirms the observations made by previous researchers that the rate of drying has an effect on increases and decreases in TA (Chkaiban *et al*., 2007), as well as metabolic processes within the berries (Bellincontro *et al*., 2004; Mencarelli and Bellincontro, 2013). If the metabolism of the berries was solely affected by the intensity of drying, then it would be expected that the TA values at all statistically similar weight loss levels between fast and slow would also be the same. The changes in patterns of TA over the course of the withering process are the result of a combination of both a concentration due to water loss, as well as a reduction due to respiration, which are both impacted by the rate and intensity of drying (Zoccatelli *et al*., 2013).

4.2 High quality total RNA is recoverable from *Vitis vinifera* 'Cabernet Franc' berry tissue at up to 132 days postharvest and 40.7 percent cluster weight loss

The extraction of intact and high quality RNA from plant tissues high in polysaccharides and polyphenols, such as grape berries, is a notoriously difficult task (Loulakakis, *et al*., 1996; Newbury and Possingham, 1977; Ma, *et al*., 2015; Yang, *et al*., 2011). Both of these compounds can interfere with the extraction of RNA, leading to low yields and/or low quality, and tend to make the isolation procedure tedious and time consuming. When tissue homogenization occurs, phenolic compounds form insoluble complexes with nucleic acids as well as proteins (Loomis, 1974; Newbury and Possingham, 1977), and polysaccharide fragments can also physically entrap nucleic acids (MacRae, 2007). The interaction of polysaccharides and polyphenols with nucleic acids leads to RNA loss during phase separation and the phenol extraction step, and thus lowers yield (Schneiderbauer, *et al*., 1991). In addition, some polyphenols (Katterman and Shattuck, 1983) and polysaccharides (Fort, *et al*., 2008; Wang and Stegemann, 2010) will coprecipitate with RNA. This contamination can interfere with downstream applications, as well as the ability to quantify RNA by spectrophotometry (Newbury and Possingham, 1977; Wang and Stegemann, 2010). The entire extraction process is further complicated in grapevine tissues due to their low RNA content (Loulakakis, *et al*., 1996).

A significant proportion of time in the present study was dedicated to the adaptation of a successful total RNA extraction technique for withered *Vitis vinifera* 'Cabernet Franc' berries. Numerous methods and protocols with various adaptations were attempted before settling on the technique described in section **2.5** of **Materials and Methods**. This protocol enabled the successful extraction of intact and high quality RNA from berries at harvest,

as well as across all fast and slow withering time points (**Figure 3.2**, **Tables 3.3** and **Table 3.4**). Successful extraction of total RNA is described by Mencarelli *et al*. (2010) from grape berries experiencing 40 percent cluster weight loss following postharvest withering, however this was under fast drying conditions lasting only 26 days. Versari *et al*. (2001) has reported successful extraction from grape berries withered for 100 days under traditional uncontrolled drying conditions. To our knowledge, the present study is the first reported successful extraction of intact high quality RNA from grape berry tissue that has undergone such a length of postharvest withering (132 days), and experienced such a level of cluster weight loss (40.7 percent). Gel electrophoresis revealed that ribosomal bands corresponding to 28S and 18S rRNA were intact and clear for all seven time points and biological replicates (21 samples) (results for seven representative samples are shown in **Figure 3.2**). RNA concentrations were high enough for downstream applications, and although the spectrophotometer absorbance ratios indicated the likely presence of some contaminants (**Table 3.3**), this did not interfere with the ability to perform RT-PCR or RNA-Seq (**Table 3.4**).

4.3 Differential gene analysis during postharvest withering at both slow and fast rates using RNA-Seq technology

To our knowledge, the present study represents the first reported transcriptome analysis of grape berries during postharvest withering which employs the use of RNA-seq technology. RNA-Seq generated high quality reads for all 21 samples in the present study, which is demonstrated by the average *phred* quality scores (*Q*-scores) obtained (**Table 3.4**). *Phred* scores estimate the confidence in a base-call, and scores are assigned on a logarithmic scale (Patton *et al*., 2006). A *phred* quality score of 40 is associated with a

base-call accuracy of 99.99 percent and a probability of 1 in 10,000 times of being incorrect (Richterich, 1998). A *phred* quality score of 30 is assigned to a base-call having a probability of being incorrect 1 in 1,000 times with an accuracy of 99.9 percent (Ewing and Green, 1998; Voelkerding *et al*., 2009). Since all samples in the present study returned average *phred* quality scores of either 35 or 36 (**Table 3.4**), these values indicate that the data is of high quality and reliable (Ewing and Green, 1998; Richterich, 1998; Voelkerding *et al*., 2009).

Between 58 to 75 percent of the total number of raw reads for all 21 samples aligned to the *V. vinifera* reference genome (NCBI *Vitis vinifera* Annotation Release 101) using Bowtie software, Version 1.1.2 (Langmead *et al*., 2009) (**Table 3.4**). The number of raw reads which aligned to a protein-coding region in the *V. vinifera* reference genome was determined using STAR software, Version 2.5 (Dobin *et al*., 2013) (**Table 3.4**). Although replicate #3 of FWII had 7,363,466 reads which aligned to a protein-coding region, this was a relatively low proportion of the 40,389,093 raw read total. There are a number of possible explanations for this result, including low quality or degraded RNA and/or genomic DNA contamination. With respect to RNA degradation, intact ribosomal bands were observed on an agarose gel following gel electrophoresis in all sequenced samples, including FWII replicate #3, which is shown in **Figure 3.2**. This sample also returned an average *phred* quality score of 36 (**Table 3.4**) and passed all additional quality control tests following RNA-Seq, and thus RNA degradation was not a concern. With respect to DNA contamination, PCR was performed as a control measure without reverse transcriptase, using RNA from all time points as a template, along with primers designed for the ORF of a lipid transfer protein (**2.6, RNA Sequencing**). The absence of bands on an agarose gel

following electrophoresis indicated that DNA contamination was unlikely. Another explanation may be related to the library preparation, which involved an enrichment of the mRNA fraction of total RNA (**2.6, RNA Sequencing**). Total RNA usually contains over 90 percent ribosomal RNA (rRNA) and only 1 to 2 percent mRNA (Conesa *et al*., 2016). The enrichment process does not completely eliminate other types of RNA such as rRNA, and this accounts for a proportion of the raw reads that are sequenced during RNA-Seq but do not align to protein-coding sequences (Dündar *et al*., 2015; Raz *et al*., 2011). As RNA was of high quality and DNA contamination was unlikely, a probable explanation is a failure of the library mRNA enrichment process to remove a large proportion of other types of RNA, resulting in these also being sequenced and thus included in the raw read counts for replicate #3 of FWII (Dündar *et al*., 2015; Raz *et al*., 2011) (**Table 3.4**).

Differential expression of genes was observed at all sampling time points throughout the drying process, including up to the end of fast and slow withering (**Figure 3.4**). Previous studies have shown that berries are still transcriptionally active at advanced stages of drying, including up to 32.7 percent berry weight loss (Fasoli *et al*., 2012; Zamboni *et al*., 2008), and 90 days postharvest withering (Fasoli *et al*., 2012; Rizzini *et al*., 2009; Zamboni *et al*., 2010). Observations in the present study are consistent with these results, with 6,525 DE genes at the end of slow withering, and 3,082 DE genes at the end of fast withering (as compared to harvest) (**Figure 3.4**), where grape clusters lost an average of 40.7 and 31.2 percent of their fresh weights, respectively (**Table 3.1**). In the present study, the number of DE genes was affected by the intensity of withering and this amount increased as weight loss was enhanced from WI to WII for both fast and slow drying (**Figure 3.4**). Using a microarray analysis, Rizzini *et al*. (2009) also observed an

increase in the number of DE probes as withering intensity increased from 10 to 30 percent weight loss in both fast and slow drying conditions.

PCA of RNA-Seq data for all time points demonstrated a clear separation of fast from slow withering along principal component 1 (PC1) (**Figure 3.3**). This result indicates that berries from the fast dry time points as a whole were transcriptionally different from those from the slow dry time points. Not only did fast withering time points segregate together away from slow (**Figure 3.3**), but those from each drying treatment were also clustered within close proximity of each other, indicating that they were more closely related together as a group. The fast withering time points were located quite close to the harvest time point along PC1, suggesting that fast dry berries were more transcriptionally similar to berries from the harvest time point (**Figure 3.3**). This observation is supported by the numbers of DE genes reported for fast dry time points, as compared to those from slow, keeping in mind that differential expression is calculated as compared to harvest (**Figure 3.4**). At each fast dry time point the number of DE genes was far below the numbers observed for slow dry (**Figure 3.4**). FWI had the lowest number of DE genes at 1,706, and this time point was located the closest to harvest on the PCA plot (**Figure 3.3**).

Within each of the time points, biological replicates were grouped closely on the PCA plot for FWI and FWII, as well as for SWI (**Figure 3.3**), meaning that at each of these individual time points during withering, the majority of berries were all in similar transcriptional states. In comparison, the biological replicates for FWIII, SWII and SWIII displayed greater variation (**Figure 3.3**), suggesting that these berries may have experienced increased transcriptional variability. These time points all represented an average of over 30 percent weight loss (**Figure 3.1** and **Table 3.1**). It is possible that not

all berries on each cluster were experiencing the same intensity of dehydration, and therefore the stress response may not have been completely uniform from berry to berry. These time points were also not distinctly separate from earlier time points on the PCA plot (**Figure 3.3**). The close overlap of FWIII with FWII, as well as between SWII and SWIII (**Figure 3.3**), indicated that these time points were similar on a transcriptional level. Indeed, the levels of DE genes between WII and WIII in both fast and slow withering were quite similar (**Figure 3.4**), with a difference of only just over 200 genes. A comparison of differential regulation between only WII and WIII revealed that there were no genes considered DE between them for both slow and fast treatments. The Venn diagrams in **Figure 3.5** also demonstrate that WII and WIII for both slow (**A**) and fast (**B**) treatments shared the highest amount of DE genes in common, beyond any other two time point comparisons.

Overall, slow withering triggered a stronger transcriptional response, with a higher number of DE genes identified (**Figure 3.4**), compared to those found at each of the fast drying time points. This result is consistent with observations by Rizzini *et al*. (2009) using microarray analysis. In the present study, the highest number of DE genes in slow dry was 6,772, which was observed at SWII, and the highest number for fast dry was 3,082 at FWIII (**Figure 3.4**). The greater number of DE genes associated with slow withering is likely due in part to the longer time period that the berries have to respond and adapt to the drying process (Zoccatelli *et al*., 2013). The improved berry structure which has been related with withering temperatures of 10 ^{o}C or under is likely also a factor, as cells are able to function at an improved capacity if their overall structure is maintained (Bellincontro *et al*., 2009).

When observing the proportion of DE genes common to all three withering time points, this amount was higher for the slow treatment as compared to fast (**Figure 3.5** and **Table 3.5**). These results are consistent with observations made by Rizzini *et al*. (2009). Those DE genes which were commonly regulated represented a minimum of 56 or 38 percent of the total DE genes at any given time point for slow and fast withering, respectively. The observation that a larger percentage of the DE genes at each slow drying time point were common to the slow withering process as a whole (**Figure 3.5** and **Table 3.5**) is reinforced by the PCA plot (**Figure 3.3**). The PCA plot demonstrates that the fast time points were much more separated out from each other and thus had more variability transcriptionally, as compared to the slow time points, which were not as individually distinct (**Figure 3.3**). Interestingly, of the DE genes common to all slow or to all fast time points, the majority are up-regulated for slow withering and for fast withering the majority are down-regulated. A similar observation was reported by Rizzini *et al*. (2009) for 'Raboso Piave' grapes dried at fast and slow rates up to 10 and 30 percent cluster weight loss.

When comparing DE genes at each individual time point between fast and slow withering (FWI verses SWI, etc.), the total amount of common DE genes was affected by drying intensity (weight loss), and this number increased from WI to WII and finally WIII (**Figure 3.6** and **Table 3.5**), which is consistent with microarray observations by Rizzini *et al*. (2009). For all individual time point comparisons in the present study, the majority of common DE genes between fast and slow withering were always up-regulated (**Table 3.5**), however this was the case only at 30 percent cluster weight loss in the study by Rizzini *et al*. (2009).

A comparison of the DE genes at all six withering time points (SWI, SWII, SWIII, FWI, FWII, FWIII) revealed that there were 432 genes which were common to every one of these samples (**Figure 3.7** and **Table 3.5**). As these 432 genes were common across all intensities of withering and weight loss levels, as well as between different rates of drying, these genes are likely characteristic of the withering process as a whole.

4.4 Slow withering invokes a diverse metabolic transcriptional reaction and fast withering primarily generates responses to abiotic and biotic stress

The present study investigated gene ontology (GO) terms that were overrepresented in several sets of DE genes. Overrepresented GO terms were determined for each of the slow and fast withering time points individually (**Tables 3.6** and **3.7**), as well as for those up-regulated DE genes common to all time points of a specific drying treatment (**Tables 3.8** and **3.9**) and finally for those DE genes common to all six time points sampled (**Figures 3.8, 3.9** and **3.10**).

By investigating those overrepresented GO terms associated with the DE genes common to all time points of a specific drying treatment, (for example common to SWI, SWII and SWIII), insight is provided into those responses that characterize either the slow drying or the fast drying process (**Tables 3.8** and **3.9**). There was a clear difference between the distribution of commonly up-regulated DE genes when comparing slow and fast drying as a whole. The slow withering process invoked a much higher number of up-regulated DE genes common to each time point as compared to fast dry (**Figure 3.4**), and this corresponded to a much higher number of overrepresented GO terms (**Tables 3.8** and **3.9**). The up-regulated DE genes common to slow drying were associated with 49 significant GO terms (**Table 3.8**), while only 12 were significant in the fast dry (**Table 3.9**). Of these

12 significant fast dry GO terms, nine were also significant for the slow dry. The only fast withering terms not significantly overrepresented also in slow withering were response to abscisic acid stimulus, second-messenger-mediated signaling and calcium-mediated signaling (**Table 3.9**). The overrepresented categories for the fast withering process included 10 response terms and two signaling categories (**Table 3.9**). However, the slow withering process included up-regulation of several metabolic and biosynthetic processes, such as phenylpropanoid and cellular aromatic compound metabolism and biosynthesis categories, regulation of various responses, also enzyme and transcription factor activity, and a vast array of diverse abiotic and biotic response reactions (**Table 3.8**). Similar slow withering responses have been suggested in previous genomic studies (Zamboni *et al*., 2010; Zamboni *et al*., 2008). The time factor associated with slow withering likely provided an opportunity for biosynthesis and diverse metabolic responses to continue (Bellincontro *et al*., 2016; Zoccatelli *et al*., 2013), however fast drying revealed a lack of GO terms in these areas when considering common DE genes across all time points of each treatment (**Table 3.9**).

With respect to each individual time point, **Tables 3.6** and **3.7** show how the distribution of DE genes associated with overrepresented GO terms changed over time for slow and fast withering. Both the intensity and the rate of withering affected the overrepresented GO terms and the association of DE genes with functional categories (**Tables 3.6** and **3.7**). The response to stimulus category and a large number of its associated child terms were the most represented at every time point across both fast and slow. The distribution of DE genes in these response categories remained fairly consistent as drying intensity increased. WII and WIII in both treatments shared the most GO terms in common,

and in all cases these shared terms had similar associated percentages of DE genes. This observation supports the previously discussion suggesting that these time points appear to have shared similar transcriptional states (**4.3, Discussion**). For slow withering, the WII time point was associated with the greatest number of overrepresented GO terms (**Table 3.6**), and for fast withering this time point was the WIII (**Table 3.7**). The clusters at both of these time points (SWII and FWIII) had just sustained a little over a 30 percent weight loss (**Table 3.1**). Overall, the GO terms with the largest percentage of associated DE genes were cell and cell part (72 and 72.6 percent), which were found only in slow drying at WII and WIII under the cellular component root category (**Table 3.6**). The significant child terms associated with these categories included plasma membrane and chloroplast, which were overrepresented at all slow dry time points. All of the cellular component terms noted for slow drying were absent from fast dry; however, at FWIII two terms under this root category became significant, plastid and plastid part, with a small percentage of associated DE genes (11.7 and 5 percent) (**Table 3.7**). The majority of significant terms associated with fast withering were related to a response or reaction of the cell to stress and stimuli. These terms included an intracellular signalling cascade, which was not a significant term at any of the slow withering time points (**Tables 3.6** and **3.7**). The diverse GO terms associated with the slow drying process are likely representative of senescence occurring in the berries due to the extended duration of the postharvest withering (Bellincontro *et al*., 2016; Zoccatelli *et al*., 2013).

Finally, investigating the overrepresented GO terms which were common to all time points, regardless of drying rate or intensity of weight loss, can provide insight into those functional categories which are characteristic of postharvest withering as a whole. Of

the 432 DE genes common to all six drying time points (**Figure 3.7**), there was almost a split between those either up- or down-regulated (202 and 230), with slightly more down-regulated genes (**Table 3.5**). Despite the close numbers for common up- and down-regulated genes, the up-regulated ones were associated with only 12 overrepresented terms (**Figure 3.8**), while the down-regulated were associated with 69 (those terms corresponding to $\geq$ 5 percent of DE genes are shown in **Figures 3.9** and **3.10**). Common to all time points was the up-regulation of genes associated with response to stimulus and these were the only overrepresented GO terms (**Figure 3.8**). Response to abiotic and biotic stimuli, as well as response to stress were a common postharvest withering responses, independent of rate or intensity of drying (**Figure 3.8**). Using a microarray approach, Rizzini *et al*. (2009) also found that response to biotic and abiotic stimuli was a common functional category for DE genes in both slow and fast drying, up to a 30 percent weight loss.

The overrepresented GO terms associated with genes down-regulated at all time points fell under both the biological process (**Figure 3.9**), as well as the cellular component root categories (**Figure 3.10**). The majority of terms were associated with a down-regulation of responses related to light, photosynthesis and the chloroplast thylakoid membrane (**Figure 3.9** and **3.10**). Zamboni *et al*. (2008) demonstrated a down-regulation of a photosystem I reaction centre subunit N chloroplast precursor during postharvest withering, which did not exhibit any down-regulation during on-plant withering. A study conducted in *Arabidopsis thaliana* has shown that the photosystem I reaction centre subunit N chloroplast precursor is located in the chloroplast thylakoid membrane (Zybailov *et al*., 2008). In the present study, there was also a down-regulation of genes associated with a GO term for pigment metabolic process (**Figure 3.9**). Taken together, these observations

may suggest the degradation or modification of carotenoids, which occurs in plants during senescence and stress (Biswal, 1995; Lashbrooke *et al*., 2013).

Carotenoids are pigments which are typically associated with chloroplast membranes, as well as other photosynthetic membranes of plastids, where they participate in light reactions (Lashbrooke *et al*., 2013). In the present study, postharvest withering was carried out in continuous darkness, which was interrupted only during sampling. Continuous darkness is known to induce senescence and results in the degradation of both carotenoids and chlorophyll in attached and detached leaves (Biswal *et al*., 1983; Biswal and Mohanty, 1976). In addition, light has been shown to retard the loss of carotenoids during senescence (Biswal, 1995). A decline in carotenoid content during fast postharvest withering has been demonstrated by Chkaiban *et al*. (2007) in both controlled and uncontrolled conditions for 'Gewürtztraminer' grapes. Carotenoids are precursors to C_{13}-norisoprenoids, which are responsible for a number of the volatile aroma compounds found in wine, and often convey floral, spicy and fruity characters (D'Onofrio, 2013; Lashbrooke *et al*., 2013). As such, possible carotenoid degradation during the postharvest withering process may have implications in the resulting wine aroma. During slow postharvest withering, the amount of total C_{13}-norisoprenoids has been shown to increase in 'Cesanese' grape berries (Centioni *et al*., 2014). In addition, two categories of C_{13}-norisoprenoids have been reported to increase in wines made from slow withered 'Corvina' and 'Rondinella' grapes (Bellincontro *el al*., 2016). An increase in transcripts of carotenoid cleavage dioxygenase 1 (*CCD1*) has been shown during slow and fast postharvest withering up to a peak at 20 percent weight loss in 'Aleatico' berries (Cirilli *et al*., 2012). This enzyme is

involved in generating norisoprenoids from different carotenoid substrates (Cirilli *et al*., 2012).

Overrepresented GO terms for down-regulated genes common across all time points also included those associated with flavonoid metabolic processes (**Figure 3.9**) and the cell wall (**Figure 3.10**). Previous studies have demonstrated the differential expression of genes encoding for enzymes involved in cell wall metabolism and modification in berries undergoing slow postharvest withering (Fasoli *et al*., 2012; Zamboni *et al*., 2008; Zoccatelli *et al*., 2013). These studies reported some cell wall genes which were down-regulated (Zamboni *et al*., 2008) and others which were up-regulated (Fasoli *et al*., 2012; Zoccatelli *et al*., 2013).

4.5 Differences and similarities between slow and fast withering expression patterns for several genes of interest

The expression patterns associated with a selection of several genes of interest were investigated. As previously mentioned, these genes were considered to be of interest because they are known to be involved in abiotic stress responses in plants, and/or their behaviour has been investigated in previous postharvest *appassimento* studies conducted in Italy. All genes contained in the *Vitis vinifera* reference genome corresponding to the following were examined: CBF transcription factor, WRKY transcription factor, MYB transcription factor, phenylalanine ammonia lyase, chalcone synthase, flavonol synthase, UDP-glucose:flavonoid 3-O-glucosyltransferase, stilbene synthase, dehydrin, alcohol dehydrogenase and trehalose-phosphate phosphatase.

In all cases, for each gene investigated there were always a greater number exhibiting differential expression in slow withering as compared to fast withering. Slow drying also displayed unique up-regulation responses for two genes of interest, *CBF3* (**Figure 3.11**) and a dehydrin (**Figure S3**), that were not observed in the fast treatment. A transient up-regulation at WI and WIII for *CBF3* occurred in slow withering, however there was no differential expression associated with fast (**Figure 3.11**). Accumulation of *VvCBF3* transcripts has been shown to occur in response to drought stress and low temperature (4 ^{o}C) treatment in young leaves of both *V. vinifera* and *V. riparia* (Xiao *et al*., 2006). Slow withering in the present study also induced the accumulation of transcripts coding for a dehydrin, while fast withering was not associated with any changes in dehydrin gene expression (**Figure S3**). Dehydrins are accumulated in plants in response to cold and dehydration stress (Close, 1997), and the up-regulation of a dehydrin during slow postharvest withering has also been observed by Zamboni *et al*. (2008). Since both dehydrins and *CBF3* are also accumulated in response to cold (Close, 1997; Xiao *et al*., 2006), the low average temperature (6 ^{o}C) associated with slow drying in the present study could be an explanation for their up-regulation in this withering treatment (**Figures 3.11** and **Figure S3**).

Both WRKY and MYB transcription factors (TFs) are accumulated in response to stress in plants (Martin and Paz-Ares, 1997; Pandey and Somssich, 2009; Ülker and Somssich, 2004). A large number of these TFs were up-regulated in both slow and fast drying in the present study (**Figures 3.12, 3.13** and **S1**) indicating their likely involvement in the dehydration stress response during postharvest withering. The up-regulation of 30 *WRKY* genes during slow drying (**Figure 3.12**) and 11 during fast (**Figure 3.13**) is

consistent with observations by Zamboni *et al*. (2008), who reported the up-regulation of a transcript during withering which shows homology to the WRKY6 transcription factor from *Nicotiana attenuata*. Consistent with the up-regulation of *MYB* TFs in the present study, two previous postharvest withering transcriptome studies in grapes have observed an up-regulation in *MYB* homologues (Bonghi *et al*., 2012; Zamboni *et al*., 2008).

The expression of a number of genes involved in phenylpropanoid biosynthesis were investigated in the present study. Numerous flavonoids, along with other phenylpropanoids such as stilbenes, are involved in abiotic and biotic stress responses (Martin and Paz-Ares, 1997). As such, gene expression during withering was explored for phenylalanine ammonia lyase (PAL) (**Figure 3.14**), chalcone synthase (CHS) (**Figure 3.15**), flavonol synthase (FLS) (**Figure 3.16**), UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) (**Figure S2**) and stilbene synthase (STS) (**Figures 3.17** and **3.18**). Previous research has demonstrated an increase in *PAL* transcripts (Bonghi *et al*., 2012; Rizzini *et al*., 2009; Tonutti *et al*., 2004; Zamboni *et al*., 2008) and *STS* transcripts (Bonghi *et al*., 2012; Mencarelli *et al*., 2010; Tonutti *et al*., 2004; Versari *et al*., 2001; Zamboni *et al*., 2008) during fast and slow withering treatments, and results in the present study were consistent with these observations. There was a strong up-regulation of *PAL* and *STS* by WI in slow withering (**Figures 3.14A** and **3.17**). A large number of STS genes were also up-regulated in fast drying, but the fold change values were not as high as observed in slow withering, and up-regulation in fast drying was delayed until WII (**Figure 3.18**). Only three *PAL* genes were up-regulated in fast drying and a similar delay in expression until WII was observed (**Figure 3.14B**). Consistent with these observations, Tonutti *et al*. (2004) also reported a rate effect associated with a delay in the timing of up-

regulation in fast drying for *PAL* and *STS* as compared to slow drying. Postharvest withering may induce a general activation of the phenylpropanoid pathway, due to an increase in *PAL* transcripts (Bonghi *et al*., 2012). In the present study, the increase in *PAL* transcripts is much more pronounced in slow drying due to the earlier timing, greater number of induced genes and higher fold change in expression levels as compared to fast (**Figures 3.17** and **3.18**). The up-regulation of *STS* appears to be characteristic of the postharvest withering response whether fast or slow. The fast withering process limits the amount of time for metabolic processes to occur due to its speed. The additional delay observed in the activation of key genes in the phenylpropanoid biosynthetic pathway during fast drying (**Figures 3.14B** and **3.18**) suggests even less opportunity for associated secondary metabolic processes to take place.

Although postharvest withering appears to induce a general activation of the phenylpropanoid pathway (**Figure 3.14**) as well as the up-regulation of stilbene production (**Figures 3.17** and **3.18**), the various classes of polyphenols may be affected in different ways. MYBA TFs and UFGT are both involved in the biosynthesis of anthocyanins, and in the present study *MYBA* genes showed no change in differential expression in fast or slow drying. In addition, *UFGT* was not differentially expressed at any of the slow withering time points and was down-regulated transiently at WII in the fast withering treatment (**Figure S2**). These results indicate that there is likely not an increase in anthocyanin biosynthesis during controlled fast or slow postharvest withering in 'Cabernet Franc' grapes. Two other genes involved in flavonoid biosynthesis, *CHS* and *FLS*, show both a down-regulation of genes and a majority down-regulation of genes, respectively, in fast withering by the WI time point (**Figures 3.15B** and **3.16B**). In slow withering the

results are similar, with a majority of *CHS* genes and half of the *FLS* genes down-regulated by WI (**Figures 3.15A** and **3.16A**). Bonghi *et al*. (2012) have shown an induction of *FLS* during withering, however the study was conducted through the use of microarray, which selects for individual genes, rather than demonstrating the behaviour of all *FLS* genes, as is the case in RNA-Seq analysis. Indeed, there was an up-regulation of some *FLS* genes in the present study (**Figure 3.16**), however the majority exhibiting differential expression were down-regulated in both fast and slow withering. With respect to *CHS*, similar to the present study (**Figure 3.15**), down-regulation has been observed by other researchers (Bonghi *et al*., 2012), and suggests a general suppression of the flavonoid branch of the phenylpropanoid pathway. This suggestion is further supported by the down-regulation or no change in expression of anthocyanin-related genes also observed by previous researchers (Bonghi *et al*., 2012; Rizzini *et al*., 2009; Tonutti *et al*., 2004), as well as by the down-regulation of genes associated with the GO term for flavonoid metabolic process for both slow and fast drying in the present study (**Figure 3.9**).

The various isogenes of alcohol dehydrogenase (ADH) demonstrate different behaviour in the literature during postharvest withering in grape berries (Cirilli *et al*., 2012; Rizzini *et al*., 2009). In the present study, differentially expressed *ADH* genes were split in both fast and slow dry, with half up-regulated and half-down regulated (**Figure 3.19**). Previous studies have reported on only one or two of these genes, as they were not conducted with RNA-Seq, and the literature reports an up-regulation of an *ADH* gene (Cirilli *et al*., 2012) and also a down-regulation of another (Rizzini *et al*., 2009). Rizzini *et al*. (2009) observed a down-regulation of a putative alcohol dehydrogenase 7 gene in both fast and slow withering, which is consistent with the behaviour of one particular

ADH7 gene (NP_001268090.1) from the present study (**Figure 3.19**). However, two additional alcohol dehydrogenase 7 genes were also up-regulated in the present study in slow withering. RNA-Seq is a powerful tool that allows for an examination of the behaviour of all identified genes coding for a specific protein, rather than a limited study of a pre-selected gene copy (Dündar *et al*., 2015). In the case of *ADH*, it appears that some isogenes may be involved in the withering response, however for others this may not be the case (**Figure 3.19**), or alternatively expression may occur at different weight loss percentages beyond those represented by the sampling time points in the present study. This observation is supported by the distinct expression patterns and likely unique metabolic roles of different *ADH* isogenes during grapevine development (Tesnière and Verriès, 2000).

Differential expression of trehalose-phosphate phosphatase (*TPP*) during withering revealed a rapid and strong up-regulation of several genes by the first time point (WI) in both fast and slow drying, and these genes remained up-regulated through WII and WIII (**Figure 3.20**). These results are consistent with the up-regulation of a *TPP* transcript reported by Zamboni *et al.* (2008) during slow withering, and suggest that trehalose may be accumulated in grape berries during postharvest withering and may act as an osmoprotectant by assisting in the modulation of dehydration stress (Crowe *et al*., 2001),

Of all of the genes of interest differentially expressed which code for the proteins discussed, there were 11 which followed similar patterns in their regulation (**Table 3.10**). These included the up-regulation of two *TPP* genes and two *STS* genes at all time points, as well as the down-regulation of a *FLS* and a *CHS* gene at all time points. It is interesting to find 11 genes which behave in the same manner in both drying treatments, but it is also

important to note that of all the genes investigated there were over 150 that did not behave similarly. Although there appear to be some fundamental genetic responses characteristic of the postharvest withering process, which are common independent of rate and intensity, there are far more differences between slow and fast withering, when considering the responses of those genes investigated in the present study.

4.6 Low levels of fungal transcripts were associated with slow and fast withering

The alignment rates of the raw reads to the *Botrytis cinerea* genome (B05.10 NCBI Genbank) from all sampling time points and replicates, for both fast and slow withering, were all less than 10 percent and showed no significant differences (**Table 3.11**). Due to the fact that a number of sequences aligned exactly to the reference genome, the presence of fungus at all time points and in both drying treatments can be assumed.

The production of STS and PAL proteins in grape cells is known to occur upon exposure to *Botrytis cinerea* cell wall fragments (Liswidowati *et al.*, 1991). It is interesting to note that although the levels of fungus were the same across fast and slow drying (**Table 3.11**), the responses of both *STS* and *PAL* were not identical in gene amount, timing or fold change (**Figures 3.14, 3.17** and **3.18**). This observation indicates that although the presence of *Botrytis cinerea* may play a role in the expression of *STS* and *PAL*, the rate of withering is still a significant factor in the up-regulation of both of these genes.

4.7 Conclusions and future work

The present study describes the use of RNA-Seq to examine transcript levels and differential gene expression in *Vitis vinifera* 'Cabernet Franc' grape berries over time, during both slow and fast postharvest *appassimento* withering. Withering was successfully

achieved through the use of a fully controlled novel air flow system, allowing for both a fast drying period of 20 days, as well as a slow drying period lasting 132 days (**Figure 3.1**). Despite the stress and weight loss associated with postharvest withering, grape berries showed incredible resilience and continued to metabolize and react transcriptionally during the process (**Figure 3.4**), even at advanced stages of drying (40.7 percent weight loss) and following months of separation from the vine (132 days).

The number of differentially expressed genes during withering was affected by dehydration stress and increased along with the intensity of drying, up to just over 30 percent cluster weight loss in both slow and fast treatments (**Figure 3.4**). The intensity and rate of drying affected berry composition (**Table 3.1**), as well as the specific gene ontology categories associated with differentially expressed genes (**Tables 3.6, 3.7, 3.8** and **3.9**). Slow withering was associated with a much higher total number of differentially expressed genes at every time point (**Figure 3.4**), as well as with the up-regulation of higher numbers of specific genes investigated, as compared to fast (**3.6, Results**). Not only did slow withering have a larger number of differentially expressed genes, but the majority of these were up-regulated, however in fast drying the majority were down-regulated (**Figure 3.4**). The up-regulated genes common across slow withering time points were associated with a large number of gene ontology categories, including response to abiotic and biotic stimuli, but also metabolic and biosynthetic processes, as well as regulation of enzyme activity and many more (**Table 3.8**). Those commonly up-regulated genes during fast drying time points were not associated with metabolic or biosynthetic processes, but rather only with gene ontology terms relating to response to abiotic and biotic stimuli (**Table 3.9**). Principal component analysis supported this observation (**Figure 3.3**) and revealed that fast

withering samples clustered closer to harvest than those from slow withering, indicating that on a transcriptional level, the berries from fast drying samples were more similar to those at harvest.

Results from the present study suggest that the time associated with the extended withering period during slow drying provided an opportunity for the berries to gradually adjust to the water stress and to continue to carry out metabolic reactions. The present study demonstrates that most differential gene expression during fast withering was associated primarily with a reaction response, rather than metabolic or biosynthetic processes (**Tables 3.7** and **3.9**). During fast withering, there was a concentration of sugars and a reduction in titratable acidy due to respiration (**Table 3.1**), however by investigating the gene ontology terms associated with those genes differentially expressed, it appears that the berries were focused almost entirely on reacting to the intense and rapid dehydration stress (**Tables 3.7** and **3.9**). The rapid process of fast withering is associated with a breakdown in cellular structure, while the tissue integrity during slow withering remains high (Bellincontro *et al*., 2009), which is likely a key factor contributing to the continuation of biosynthetic and secondary metabolic processes during the lengthy slow drying period (**Tables 3.6** and **3.8**).

The last two time points in both slow and fast withering in the present study were still transcriptionally reactive (**Figure 3.4**), however they were similar in terms of transcriptional states (**Figure 3.3**). For slow withering, both time points represented over 30 percent weight loss and greater than 100 days of drying (**Figure 3.1**), and achieving these together is the goal of the traditional uncontrolled *appassimento* process (Accordini, 2013; Paronetto and Dellaglio, 2011; Tonutti *et al*., 2004). The present study demonstrates that reaching these parameters was associated with a specific transcriptional state, which

was still maintained at up to 40.7 percent weight loss and 132 days postharvest withering. For the winemaker pursuing a slow dry *appassimento* wine style, once achieving these parameters this information could produce two different responses; if the target ^{o}Brix has not yet been met, the grapes could be allowed to continue to dry slowly for longer than 100 days, or the drying rate could be increased in order to meet the target ^{o}Brix in less time. As fast drying at higher temperatures are associated with increased oxidation and changes in volatile profiles (Cirilli *et al*., 2012; Mencarelli and Bellincontro, 2013), employing a rapid drying rate after 100 days of withering will likely introduce further changes to the berry composition.

When considering the expression patterns of genes coding for a specific protein, for those investigated in the present study there was always a higher amount differentially expressed in slow withering (**3.6, Results**). There were some common responses between drying rates (**Table 3.10**) and both slow and fast withering were associated with an up-regulation of numerous WRKY and MYB transcription factors (**Figures 3.12, 3.13** and **S1**), as well as stilbene synthase (**Figures 3.17** and **3.18**), phenylalanine ammonia lyase (**Figure 3.14**) and trehalose-phosphate phosphatase (**Figure 3.20**). Also common was the behaviour of genes associated with anthocyanin biosynthesis, as there was no change in expression for *MYBA* genes, and UDP-glucose:flavonoid 3-O-glucosyltransferase was down-regulated transiently in fast withering (**Figure S2**) and showed no change in expression in slow. Slow withering was also associated with some unique responses, such as the observation of an up-regulation of a dehydrin (**Figure S3**) and the *CBF3* gene (**Figure 3.11A**).

To our knowledge, the present study demonstrates the first reported use of RNA-Seq to study changes in transcript abundance during grape postharvest withering, as well as the first reported successful extraction of intact RNA from berries sustaining up to 40.7 percent cluster weight loss after 132 days drying. RNA-Seq is a powerful tool and has significant advantages over microarrays in that all protein-coding transcripts at a given time point are captured, and the behaviour of all genes in the reference genome coding for a particular protein can be investigated (Raz *et al*., 2011). Microarrays and similar technologies provide limited information as compared to RNA-Seq in that they may only focus on one or two individual genes coding for a protein, however RNA-Seq provides a vast amount of information, which is useful especially if several isogenes have different expression patterns (Sweetman *et al*., 2012). The analysis of differential gene expression through RNA-Seq in the present study revealed some characteristic genetic responses to the postharvest withering process in 'Cabernet Franc' grapes, which are common independent of rate and intensity. However, there were far greater differences observed between the differential gene expression responses when comparing slow and fast withering. The results of the present study suggest that fast withering may be associated with primarily an intense stress response with a focus on survival, leaving little time or opportunity for over-ripening processes, and slow withering may be associated with more of a senescence or over-ripening, involving diverse biosynthetic and metabolic reactions, in combination with a gradual adaptation to a less intense dehydration stress. These diverse gene expression profiles provide some insight into the molecular changes associated with water stress in fast and slow withered berries, which are both used to produce different *appassimento* wine styles.

The work presented in this thesis has generated a large RNA-Seq data set, and although the analysis presented herein has provided some insight into transcripts associated with fast and slow controlled *appassimento* drying, as well as the postharvest withering stress response as a whole, there is a wealth of information that has yet to be explored. Future work could mine this RNA-Seq data set to investigate in detail the unique gene expression responses that distinguish fast and slow drying from each other, as well as examine other metabolic pathways. In addition, the results presented in this thesis provide a foundation for further investigation that can advance and build upon gene expression insight, progressing to berry composition and finally through to wine characteristics. The ultimate goal being to determine how different *appassimento* rates affect the unique characteristics of their resulting wines. From there, additional work could begin the search for a quality marker associated with achieving desirable wine characteristics in fast and/or slow *appassimento*, which would assist the winemaker with determining the appropriate end of withering, beyond solely relying on the monitoring of ^{o}Brix levels. Such a tool would allow for a more standardized drying process that offers consistent results to the winemaker. The results presented in this thesis, along with the RNA-Seq data set, have created a valuable foundation, which will assist with future research in these directions.

References

Accordini, D. (2013). Amarone. In Mencarelli, F. and Tonutti, P. (Eds.), *Sweet, Reinforced and Fortified Wines*, Hoboken, NJ: John Wiley & Sons, Ltd., 189-203.

Akhtar, M., Jaiswal1, A., Taj, G., Jaiswal1, J.P., Qureshi, M.I., and Singh, N.K. (2012). DREB1/CBF transcription factors: their structure, function and role in abiotic stress tolerance in plants. *Journal of Genetics, 91*(3), 385-395.

Amselem, J. *et al*. (2011). Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea. PLoS Genetics, 7*(8), e1002230.

Antelmi, G., Bellincontro, A., Mencarelli, F., Nicoletti, I., and Corradini, D. (2010). How dehydration temperature and weight loss affect the biosynthesis of nutritional compounds in irrigated 'Aleatico' grape. *Acta Horticulturae, 877*, 693-698.

Artlip, T.S. and Wisniewski, M.E. (2002). Induction of Proteins in Response to Biotic and Abiotic Stresses. In Pessarakli, M. (Ed.), *Handbook of Plant and Crop Physiology*, 2nd Edition, New York, NY: Marcel Dekker, Inc., 657-679.

Barbanti D., Mora, B., Ferrarini, R., Tornielli, G.B., and Cipriani, M. (2008). Effect of various thermo-hygrometric conditions on the withering kinetics of grapes used for the production of ‘‘Amarone” and ‘‘Recioto” wines. *Journal of Food Engineering, 85,* 350-358.

Bellincontro A., De Santis, D., Botondi, R., Villa, I., and Mencarelli, F. (2004). Different postharvest dehydration rates affect quality characteristics and volatile compounds of Malvasia, Trebbiano and Sangiovese grapes for wine production. *Journal of the Science of Food and Agriculture, 84,* 1791-1800.

Bellincontro, A., Matarese, F., D’Onofrio, C.D., Accordini, D., Tosi, E., and Mencarelli, F. (2016). Management of postharvest grape withering to optimise the aroma of the final wine: A case study on Amarone. *Food Chemistry, 213*, 378-387.

Bellincontro, A., Nicoletti, I., Valentini, M., Tomas, A., De Santis, D., Corradini, D., and Mencarelli, F. (2009). Integration of nondestructive techniques with destructive analyses to study postharvest water stress of winegrapes. *American Journal of Enology and Viticulture, 60*(1), 57-65.

Biswal, B. (1995). Carotenoid catabolism during leaf senescence and its control by light. *Journal of Photochemistry and Photobiology B: Biology, 30,* 3-13.

Biswal, B., Choudhury, N.K., Sahu, P., and Biswal, U.C. (1983). Senescence of detached fern leaves. *Plant and cell physiology*, *24*(7), 1203-1208.

Biswal, U.C. and Mohanty, P. (1976). Aging induced changes in photosynthetic electron transport of detached barley leaves. *Plant and Cell Physiology, 17*(2), 323-331.

Blackman, F.F. and Parija, P. (1928). Analytic studies in plant respiration. I.-The respiration of a population of senescent ripening apples. *Proceedings of the Royal Society of Biological Sciences, 103,* 412-445.

Bonghi, C., Rizzini, F.M., Gambuti, A., Moio, L., and Chkaiban, L. (2012). Phenol compound metabolism and gene expression in the skin of wine grape (*Vitis vinifera* L.) berries subjected to partial postharvest dehydration. *Postharvest Biology and Technology, 67,* 102–109.

Castellarin, S.D., Pfeiffer, A., Sivilotti, P., Degan, M., Peterlunger, E., and Di Gaspero, G. (2007). Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under seasonal water deficit. *Plant, Cell and Environment, 30,* 1381-1399.

Centioni, L., Tiberi, D., Pietromarchi, P., Bellincontro, A., and Mencarelli, F. (2014). Effect of postharvest dehydration on content of volatile organic compounds in the epicarp of Cesanese grape berry. *American Journal of Enology and Viticulture, 65*(3), 333-340.

Chalker-Scott, L. (1999). Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology, 70*(1), 1-9.

Chervin, C., El-Kereamy, A., Roustan, J., Latche, A., Lamon, J., and Bouzayen, M. (2004). Ethylene seems required for the berry development and ripening in grape, a non-climacteric fruit. *Plant Science, 167,* 1301-1305.

Chkaiban L., Botondi, R., Bellicontro, A., De Santis, D., Kefalas, P., and Mencarelli, F. (2007). Influence of postharvest water stress on lipoxygenases and alcohol dehydrogenase activities, and on the composition of some volatile compounds of Gewürztraminer grapes dehydrated under controlled and uncontrolled thermohygrometric conditions. *Australian Journal of Grape and Wine Research, 13,* 142-149.

Cirilli, M., Bellincontro, A., De Santis, D., Botondi, R., and Colao, M.C. (2012). Temperature and water loss affect ADH activity and gene expression in grape berry during postharvest dehydration. *Food Chemistry, 132,* 447-454.

Close, T.J. (1997). Dehydrins: A commonalty in the response of plants to dehydration and low temperature. *Physiologia Plantarum, 100,* 291-296.

Conesa, A., Madrigal, P., Tarazona, S., Gomez-Cabrero, D., Cervera, A., McPherson, A., Wojciech Szcześniak, M., Gaffney, D.J., Elo, L.L., Zhang, X., and Mortazavi, A. (2016). A survey of best practices for RNA-seq data analysis. *Genome Biology, 17*(13), doi:10.1186/s13059-016-0881-8

Coombe, B.G. (1995). Adoption of a system for identifying grapevine growth stages. *Australian Journal of Grape and Wine Research, 1,* 100-110.

Corrandini, D. and Nicoletti, I. (2013). Chapter 6: Changes in Phenolic Compounds. In Mencarelli, F. and Tonutti, P. (Eds.), *Sweet, Reinforced and Fortified Wines*, Hoboken, NJ: John Wiley & Sons, Ltd, 105-118.

Cortina, C. and Culiáñez-Macia, F.A. (2005). Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. *Plant Science, 169,* 75-82

Costantini, V., Bellincontro, A., De Santis, D., Botondi, R., and Mencarelli F. (2006). Metabolic changes of Malvasia grapes for wine production during postharvest drying. *Journal of Agricultural and Food Chemistry, 54,* 3334-3340.

Cramer, G.R., Ergül, A., Grimplet, J., Tillett, R.L., Tattersall, E.A.R., Bohlman, M.C., Vincent, D., Sonderegger, J., Evans, J., Osborne, C., Quilici, D., Schlauch, K.A., Schooley, D.A., and Cushman, J.C. (2007). Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. *Functional & Integrative Genomics, 7,* 111-134.

Crowe, J.H., Crowe, L.M., Oliver, A.E., Tsvetkova, N., Wolkers, W., and Tablin, F. (2001). The trehalose myth revisited: Introduction to a symposium on stabilization of cells in the dry state. *Cryobiology 43,* 89-105.

Di Carli, M., Zamboni, A., Pe, M.E., Pezzotti, M., Lilley, K.S., Benvenuto, E., and Desiderio A. (2011). Two-dimensional differential in gel electrophoresis (2D-DIGE) analysis of grape berry proteome during postharvest withering. *Journal of Proteome Research, 10,* 429-446.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics, 29*(1), 15-21.

Dolferus, R., Peacock, W.J., and Dennis, E.S. (1994). Differential interactions of promoter elements in stress responses of the *Arabidopsis Adh* gene. *Plant Physiology, 105,* 1075-1078.

D'Onofrio, C. (2013). Changes in Volatile Compounds. In Mencarelli, F. and Tonutti, P. (Eds.), *Sweet, Reinforced and Fortified Wines*, Hoboken, NJ: John Wiley & Sons, Ltd., 91-103.

Du, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Research, 38,* W64-W70.

Dündar, F., Skrabanek, L., and Zumbo, P. (2015). Introduction to differential gene expression analysis using RNA-seq. Applied Bioinformatics Core: Well Cornell Medical College, pp 72.

Ewing, B. and Green, P. (1998). Base-calling of automated sequencer traces using *phred.* II. Error probabilities. *Genome Research, 8,* 186-194.

Fasoli, M., Dal Santo, S., Zenoni, S., Battista Tornielli, G., Farina, L., Zamboni, A., Porceddu, A., Venturini, L., Bicego, M., Murino, V., Ferrarini, A., Delledonne, M., and Pezzottia, M. (2012). The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. *The Plant Cell, 24,* 3489-3505.

Farias-Rodriguez, R., Mellor, R.B., Arias, C., and Peiia-Cabriales, J.J. (1998). The accumulation of trehalose in nodules of several cultivars of common bean *(Phaseolus vulgaris)* and its correlation with resistance to drought stress. *Physiologia Plantarum, 102,* 353-359.

Fernandez, O., Béthencourt, L., Quero, A., Sangwan, R.S., and Clément, C. (2010). Trehalose and plant stress responses: friend or foe? *Trends in Plant Science, 15,* 409-417.

Ferrandino, A. and Lovisolo, C. (2014). Abiotic stress effects on grapevine (*Vitis vinifera* L.): Focus on abscisic acid-mediated consequences on secondary metabolism and berry quality. *Environmental and Experimental Botany, 103,* 138-147.

Flanzy, C., Andre P., Benard, P., Buret, M., Chambroy, Y., and Jouret, C. (1974). Intracellular fermentation of grapes during aerobic metabolism (Fermentation intracellulaire des baies de raisin au cours de leur me´tabolisme anae´robie). *Annales de technologie agricole, 23*(4), 481-500.

Fort, F., Hayoun, L., Valls, J., Canals, J.M., Arola, L., and Zamora, F. (2008). A new and simple method for rapid extraction and isolation of high-quality RNA from grape (*Vitis vinifera*) berries. *Journal of the Science of Food and Agriculture, 88,* 179-184.

Franco, M., Peinado, R.A., Medina, M., and Moreno, J. (2004) Off-vine grape drying effect on volatile compounds and aromatic series in must from Pedro Ximenez grape variety. *Journal of Agriculture and Food Chemistry 52,* 3905-3910.

Garg, A.K., Kim, J-K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., and Wu, R.J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proceedings of the National Academy of Sciences of the United States of America, 99*(25), 15898-15903.

Goddijn, O.J.M and van Dun, K. (1999). Trehalose metabolism in plants. *Trends in Plant Science, 4*(8), 315-319.

Gresele, P., Pignatelli, P., Guglielmini, G., Carnevale, R., Mezzasoma, A.M., Ghiselli, A., Momi, S., and Violi, F. (2008). Resveratrol, at concentrations attainable with moderate wine consumption, stimulates human platelet nitric oxide production. *The Journal of Nutrition, 138*(9), 1602-1608.

Grierson, W. (2002). Role of Temperature in the Physiology of Crop Plants: Pre- and Postharvest. In Pessarakli, M. (Ed.), *Handbook of Plant and Crop Physiology*, 2nd Edition, New York, NY: Marcel Dekker, Inc., 13-33.

Grimplet, J., Deluc, L.G., Tillett, R.L., Wheatley, M.D., Schlauch, K.A., Cramer, G.R., and Cushman, J.C. (2007). Tissue-specific mRNA expression profiling in grape berry tissues. *BMC Genomics, 8,* 187-209.

Hare, P.D., Cress, W.A., and Van Staden, J. (1998). Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell and Environment 21,* 535-553.

Höll, J., Vannozzi, A., Czemmel, S., D'Onofrio, C., Walker, A.R., Rausch, T., Lucchin, M., Boss, P.K., Dry, I.B., and Bogs, J. (2013). The R2R3-MYB transcription factors MYB 14 and MYB 15 regulate stilbene biosynthesis in *Vitis vinifera*. *The Plant Cell, 25,* 4135-4149.

Hsaio, T.C. (1973). Plant responses to water stress. *Annual Review of Plant Physiology, 24,* 519-570.

Hussain, S.S., Raza, H., Afzal, I., and Kayani, M.A. (2012). Transgenic plants for abiotic stress tolerance: current status. *Archives of Agronomy and Soil Science, 58*(7), 693-721.

Katterman, F.R.H. and Shattuck, V.I. (1983). An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. *Preparative Biochemistry, 13*(4), 347-359.

Kays, S.J. (1997). Stress in Harvested Products, In Kays, S.J. (Ed.), *Postharvest Physiology in Perishable Plant Products*, Athens, GA: Exon Press, 335-408.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology, 10*(3), R25.

Lashbrooke, J.G., Young, P.R., Dockrall, S.J., Vasanth, K., and Vivier, M.A. (2013). Functional characterisation of three members of the *Vitis vinifera* L. carotenoid cleavage dioxygenase gene family. *BMC Plant Biology, 13,* 156-172.

Li, T.H. and Li, S.H. (2005). Leaf responses of micropropagated apple plants to water stress: nonstructural carbohydrate composition and regulatory role of metabolic enzymes. *Tree Physiology, 25,* 495-504.

Liswidowati, F.M., Hohmann, F., Schwer, B., and Kindl, H. (1991). Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. *Planta, 183,* 307-314.

Liu, Y., Zhou, J., and White, K.P. (2014). RNA-Seq differential expression studies: more sequence or more replication? *Bioinformatics, 30*(3), 301-304.

Lo, C-C. and Chain, P.S.G. (2014). Rapid evaluation and quality control of next generation sequencing data with FaQCs. *BMC Bioinformatics, 15,* 366-372.

Loescher, W.H. (1987). Physiology and metabolism of sugar alcohols in higher plants. *Physiologia Plantarum, 70,* 553-557.

Loomis, W.D. (1974). Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods Enzymology, 31,* 528-544.

Loulakakis, K.A., Roubelakis-Angelakis, K.A., and Kanellis, A.K. (1996). Isolation of functional RNA from grapevine tissues poor in nucleic acid content. *American Journal of Enology and Viticulture, 47*(2), 181-185.

Ma, Z., Huang, B., Xu, S., Chen, Y., Li, S., and Lin, S. (2015). Isolation of high-quality total RNA from Chinese fir (*Cunninghamia lanceolate* (Lamb.) Hook). *PLoS ONE, 10*(6), e0130234.

Martin, C. and Paz-Ares, J. (1997). MYB transcription factors in plants. *Trends in Genetics, 13*(2), 67-73.

Matton, D.P., Constabel, P., and Brisson, N. (1990). Alcohol dehydrogenase gene expression in potato following elicitor and stress treatment. *Plant Molecular Biology, 14,* 775-783.

MacRae, E. (2007). Extraction of plant RNA. In Hilario, E. and Mackay, J. (Eds.), *Methods in Molecular Biology, vol. 353: Protocols for Nucleic Acid Analysis by Nonradioactive Probes*, Totowa, NJ: Humana Press Inc., 15-24.

Mencarelli, F. and Bellincontro, A. (2013). Technology and Management of Postharvest Dehydration. In Mencarelli, F. and Tonutti, P. (Eds.), *Sweet, Reinforced and Fortified Wines*, Hoboken, NJ: John Wiley & Sons, Ltd., 51-75.

Mencarelli, F., Bellincontro, A., Nicoletti, I., Cirilli, M., Muleo, R., and Corradini, D. (2010). Chemical and biochemical change of healthy phenolic fractions in winegrape by means of postharvest dehydration. *Journal of Agricultural and Food Chemistry, 58,* 7557-7564.

Mizoi, J., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2012). AP2/ERF family transcription factors in plant abiotic stress responses. *Biochimica et Biophysica Acta, 1819,* 86-96.

Moreno, J.J., Cerpa-Calderón, F., Cohen, S.D., Fang, Y., Qian, M., and Kennedy, J.A. (2008). Effect of postharvest dehydration on the composition of pinot noir grapes (*Vitis vinifera* L.) and wine. *Food Chemistry, 109,* 755-762.

Morozova, K., Romano, A., Lonardi, F., Ferrarini, R., Biasioli, F. and Scampicchio, M. (2016). Microcalorimetric monitoring of grape withering. *Thermochimica Acta, 630,* 31-36.

Newbury, H.J. and Possingham, J.V. (1977). Factors affecting the extraction of intact ribonucleic acid from plant tissues containing interfering phenolic compounds. *Plant Physiology, 60,* 543-547.

Ohta, K., Moriguchi, R., Kanahama, K., Yamaki, S., and Kanayama, Y. (2005). Molecular evidence of sorbitol dehydrogenase in tomato, a non-Rosaceae plant. *Phytochemistry, 66,* 2822-2828.

Pandey, S.P and Somssich, I.E. (2009). The Role of WRKY Transcription Factors in Plant Immunity. *Plant Physiology, 150*(4), 1648-1655.

Paronetto L. and Dellaglio, F. (2011). Amarone: A Modern Wine Coming from an Ancient Production Technology. In Jackson, R.S. (Ed.), *Advances in Food and Nutrition Research,* Volume 63, Waltham, MA: Elsevier Inc., 285-306.

Patton, S.J., Wallace, A.J., and Elles, R. (2006). Benchmark for evaluating the quality of DNA sequencing: Proposal from an international external quality assessment scheme. *Clinical Chemistry, 52*(4), 728-736.

Pedreschi, R. and Lurie, S. (2015). Advances and current challenges in understanding postharvest abiotic stresses in perishables. *Postharvest Biology and Technology, 107,* 77-89.

Penna, P., Teixeira da Silva, J.A., and Anant, B.V. (2006). Plant Abiotic Stress, Sugars and Transgenics: A Perspective. In *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical,* Volume III, UK: Global Science Books, 86-93.

Pilcher, K.E., Gaudet, P., Fey, P., Kowal, A.S., and Chisholm, R.L. (2007). A general purpose method for extracting RNA from *Dictyostelium* cells. *Nature Protocols, 2*(6), 1329-1332.

Puhakainen, T., Hess, M.W., Mäkelä, P., Svensson, J., Heino, P., Palva, E.T. (2004). Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis*. Plant Molecular Biology, 54, 743-753.

Ramos, I.N., Silva, C.L.M., Sereno, A.M., and Aguilera, J.M. (2004). Quantification of microstructural changes during first stage air drying of grape tissue. *Journal of Food Engineering 62,* 159-164.

Raz, T., Kapranov, P., Lipson, D., Letovsky, S., Milos, P.M., and Thompson, J.F. (2011). Protocol dependence of sequencing-based gene expression measurements. *PLoS One 6*(5), e19287.

Richterich, P. (1998). Estimation of errors in ‘‘raw’’ DNA sequences: A validation study. *Genome Research, 8,* 251-259.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). *limma* powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research, 43*(7), e47.

Rizzini, F.M., Bonghi, C., Chkaiban, L., Martinelli, F., and Tonutti, P. (2010). Effects of postharvest partial dehydration and prolonged treatments with ethylene on transcript profiling in skins of wine grape berries. In Erkan, M., and Aksoy, U. (Eds.), Proceedings of the 6th International Postharvest Symposium. International Society for Horticultural Science. *Acta Horticulturae, 877,* 1099-1104.

Rizzini, F.M., Bonghi, C., and Tonutti, P. (2009). Postharvest water loss induces marked changes in transcript profiling in skins of wine grape berries. *Postharvest Biology and Technology, 52,* 247-253.

Robinson, S.P. and Davies, C. (2000). Molecular biology of grape ripening. *Australian Journal of Grape and Wine Research, 6,* 175-188.

Rolle, L., Giacosa, S., Rio Segade, S., Ferrarini, R., Torchio, F., Gerbi, V. (2013). Influence of different thermohygrometric conditions on changes in instrumental texture properties and phenolic composition during postharvest withering of ‘Corvina’ winegrapes (*Vitis vinifera* L.). *Drying Technology, 31,* 549-564.

Rontein, D., Basset, G., and Hanson, A.D. (2002). Metabolic engineering of osmoprotectant accumulation in plants. *Metabolic Engineering, 4,* 49-56.

Rorat, T. (2006). Plant dehydrins – Tissue location, structure and function. *Cellular and Molecular Biology Letters, 11,* 536-556.

Rothschild, L.J. and Mancinelli, R.L. (2001). Life in extreme environments. *Nature, 409,* 1092-1101.

Sacher, J.A. (1973). Senescence and postharvest physiology. *Annual Review of Plant Physiology, 24,* 197-224.

Schneiderbauer, A., Sandermann, H., and Ernst, D. (1991). Isolation of functional RNA from plant tissues rich in phenolic compounds. *Analytical Biochemistry, 197,* 91-95.

Sheveleva, E.V., Marquez, S., Chmara, W., Zegeer, A., Jensen, R.G., and Bohnert, H.J. (1998). Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco: high amounts of sorbitol lead to necrotic lesions. *Plant Physiology, 117,* 831-839.

Siddiqua, M. and Nassuth, A. (2011). *Vitis CBF1* and *Vitis CBF4* differ in their effect on *Arabidopsis* abiotic stress tolerance, development and gene expression. *Plant, Cell and Environment, 34,* 1345-1359.

Smirnoff, N. (1998). Plant resistance to environmental stress. *Current Opinion in Biotechnology, 9,* 214-219.

Soleas, G.J., Diamandis, E.P., and Goldberg, D.M. (1997). Resveratrol: A molecule whose time has come? And gone? *Clinical Biochemistry, 30*(2), 91-113.

Staats, M. and van Kan, J.A.L. (2012). Genome update of *Botrytis cinerea* strains B05.10 and T4. *Eukaryotic Cell, 11*(11), 1413-1414.

Sweetman, C., Deluc, L.G., Cramer, G.R., Ford, C.M., and Soole, K.L. (2009). Regulation of malate metabolism in grape berry and other developing fruits. *Phytochemistry, 70,* 1329-1344.

Sweetman, C., Wong, D.C.J., Ford, C.M., and Drew, D.P. (2012). Transcriptome analysis at four developmental stages of grape berry (*Vitis vinifera* cv. Shiraz) provides insights into regulated and coordinated gene expression. *BMC Genomics, 13,* 691-715.

Tao, R., Uratsu, S.L., and Dandekar, A.M. (1995). Sorbitol synthesis in transgenic tobacco with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase. *Plant and Cell Physiology, 36*(3), 525-532.

Tesnière, C. and Abbal, P. (2009). Alcohol dehydrogenase genes and proteins in grapevine. In Roubelakis-Angelakis, K.A. (Ed.), *Grapevine Molecular Physiology & Biotechnology,* 2nd Edition, Berlin, Germany: Springer Science, 141-160.

Tesnière, C. and Verriès, C. (2000). Molecular cloning and expression of cDNAs encoding alcohol dehydrogenases from *Vitis vinifera* L. during berry development. *Plant Science, 157,* 77-88.

Toffali, K., Zamboni, A., Anesi, A., Stocchero, M., Pezzotti, M., Levi, M., Guzzo, F. (2011). Novel aspects of grape berry ripening and post-harvest withering revealed by untargeted LC-ESI-MS metabolomics analysis. *Metabolomics, 7,* 424-436.

Tonutti, P. and Bonghi, C. (2013). Biochemistry and Physiology of Dehydrating Berries. In Mencarelli, F. and Tonutti, P. (Eds.), *Sweet, Reinforced and Fortified Wines,* Hoboken, NJ: John Wiley & Sons, Ltd., 77-90.

Tonutti, P., Tornielli, G.B., and Cargnello, G. (2004). Characterization of "territoires" throughout the production of wines obtained with withered grapes: The cases of "Terra Della Valpolicella" (Verona) and "Terra Della Valle del Piave" (Treviso) in northern Italy. *Proceedings of the International Conference on Viticultural Zoning,* Cape Town, South Africa, 314-320.

Ülker, B. and Somssich, I.E. (2004). WRKY transcription factors: from DNA binding towards biological function. *Current Opinion in Plant Biology, 7,* 491-498.

Versari, A., Parpinello, G.P., Tornielli, G.B., Ferrarini, R., and Giulivo, C. (2001). Stilbene compounds and stilbene synthase expression during ripening, wilting, and UV treatment in grape cv. Corvina. *Journal of Agricultural and Food Chemistry, 49*(11), 5531-5536.

Vigneault, C. and Goyette, B. (2002). Design of plastic container openings to optimize forced-air precooling of fruits and vegetables. *Applied Engineering in Agriculture, 18*(1), 73-76.

Voelkerding, K.V., Dames, S.A., and Durtschi, J.D. (2009). Next-generation sequencing: From basic research to diagnostics. *Clinical Chemistry, 55*(4), 641-658.

Wang, L. and Stegemann, J.P. (2010). Extraction of high quality RNA from polysaccharide matrices using cetlytrimethylammonium bromide. Biomaterials, 31(7), 1612-1618.

Wang, M., Vannozzi, A., Wang, G., Zhong, Y., Corso, M., Cavallini, E., Cheng, Z. (2015). A comprehensive survey of the grapevine VQ gene family and its transcriptional correlation with WRKY proteins. *Frontiers in Plant Science, 6*, 417-432.

Xiao, H. and Nassuth, A. (2006). Stress- and development-induced expression of spliced and unspliced transcripts from two highly similar dehydrin 1 genes in *V. riparia* and *V. vinifera*. *Plant Cell Reports, 25,* 968-977.

Xiao, H., Siddiqua, M., Braybrook, S., and Nassuth, A. (2006). Three grape *CBF/DREB1* genes respond to low temperature, drought and abscisic acid. *Plant, Cell and Environment, 29,* 1410-1421.

Xiao, H., Tattersall, E.A.R., Siddiqua, M.K., Cramer, G.R., and Nassuth, A. (2008). CBF4 is a unique member of the CBF transcription factor family of *Vitis vinifera* and *Vitis riparia. Plant, Cell and Environment, 31,* 1-10.

Yang, C., Li, F., Ji, X., and Wang, J. (2011). An effective method for RNA extraction from grapevine berry skins. *African Journal of Biotechnology, 10*(45), 9032-9035.

Yeo, E.T., Kwon, H.B., Han, S.E., Lee, J.T., Ryu, J.C., and Byun, M.O. (2000). Genetic engineering of drought-resistant potato plants by introduction of the trehalose-6-phosphate synthase (*TPS1*) gene from *Saccharomyces cerevisiae. Molecules and Cells, 10*(3), 263-268.

Yin, X., Allan, A.C., Xu, Q., Burdon, J., Dejnoprat, S., Chen, K., and Ferguson, I.B. (2012). Differential expression of kiwifruit *ERF* genes in response to postharvest abiotic stress. *Postharvest Biology and Technology, 66,* 1-7.

Zamboni, A., Di Carli, M., Guzzo, F., Stocchero, M., Zenoni, S., Ferrarini, A., Tononi, P., Toffali, K., Desiderio, A., Lilley, K.S., Pè, M.E., Benvenuto, E., Delledonne, M., and Pezzotti, M. (2010). Identification of putative stage-specific grapevine berry biomarkers and omics data integration into networks. *Plant Physiology, 154,* 1439-1459.

Zamboni, A., Minoia, L., Ferrarini, A., Tornielli, G.B., Zago, E., Delledonne, M., and Pezzotti, M. (2008). Molecular analysis of post-harvest withering in grape by AFLP transcriptional profiling. *Journal of Experimental Botany, 59*(15), 4145-4159.

Zhuang, J., Peng, R., Cheng, Z., Zhang, J., Cai, B., Zhang, Z., Gao, F., Zhu, B., Fu, X., Jin, X, Chen, J., Qiao, Y., Xiong, A., and Yao, Q. (2009). Genome-wide analysis of the putative AP2/ERF family genes in *Vitis vinifera*. *Scientia Horticulturae, 123,* 73-81.

Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd merger. *Bioinformatics, 30*(5), 614-620.

Zhao, H.W., Chen, Y.J., Hu, Y.L., Gao, Y., and Lin, Z.P. (2000). Construction of a trehalose-6-phosphate synthase gene driven by drought-responsive promoter and expression of drought-resistance in transgenic tobacco. *Acta Botanica Sinica, 42*(6), 616-619.

Zoccatelli, G., Zenoni, S., Savoi, S., Dal Santo, S., Tononi, P., Zandona, V., Dal Cin, A., Guantieri, V., Pezzotti, M., and Tornielli, G.B. (2013). Skin pectin metabolism during the postharvest dehydration of berries from three distinct grapevine cultivars. *Australian Journal of Grape and Wine Research, 19,* 171-179.

Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., and van Wijk, K.J. (2008). Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS ONE, 3*(4), e1994.

Appendix I: Supplementary Tables

Table S1 Top five up-regulated genes based on log_2 gene expression values at each slow postharvest withering time point in *Vitis vinifera* 'Cabernet Franc' berries.

Sampling time point	Gene	Accession[z]	Log_2 gene expression value
SWI	uncharacterized protein	XP_002263884.1	8.137602742
	uncharacterized protein	XP_010664490.1	7.869309105
	pectinesterase 2-like	XP_002265740.2	7.686216266
	subtilisin-like protease	XP_010661612.1	7.615555038
	ubiquitin-protein ligase PUB23-like	XP_002264761.2	7.186757584
SWII	E3 ubiquitin-protein ligase PUB23-like	XP_002264761.2	9.397007322
	G-type lectin S-receptor-like serine/threonine-protein kinase RLK1	XP_002275592.1	8.835599723
	uncharacterized protein	XP_010664490.1	8.701967435
	putative serine/threonine-protein kinase isoform X2	XP_010658416.1	8.529776551
	26S protease regulatory subunit 10B homolog A-like	XP_010660161.1	8.518810096
SWIII	uncharacterized protein	XP_002263884.1	8.997451126
	E3 ubiquitin-protein ligase PUB23-like	XP_002264761.2	8.88660991
	G-type lectin S-receptor-like serine/threonine-protein kinase RLK1	XP_002275592.1	8.609712067
	pathogenesis-related protein STH-21-like	XP_002269128.1	8.60481421
	probable mannitol dehydrogenase	XP_002263847.1	8.30678605

[z] Genscope accession number (NCBI *Vitis vinifera* Annotation Release 101 genome)

Table S2 Top five up-regulated genes based on log_2 gene expression values at each fast postharvest withering time point in *Vitis vinifera* 'Cabernet Franc' berries.

Sampling time point	Gene	Accession[z]	Log_2 gene expression value
FWI	basic endochitinase	XP_002269727.2	5.500908976
	probable terpene synthase 9	XP_002275106.1	5.102376796
	uncharacterized protein	XP_002275464.1	5.016314728
	uncharacterized protein	XP_002274919.1	4.939880343
	chitinase 5	XP_002275534.1	4.899444282
FWII	uncharacterized protein	XP_002274844.1	7.518733069
	carbonic anhydrase 2-like	XP_010663173.1	7.18046614
	germin-like protein subfamily 1 member 15	XP_002284682.1	6.636439523
	uncharacterized protein	XP_002272652.1	6.464262613
	glutathione S-transferase U8	XP_010650966.1	5.988602653
FWIII	uncharacterized protein	XP_002274815.2	8.204718327
	carbonic anhydrase 2-like	XP_010663173.1	7.265060759
	uncharacterized protein	XP_002272652.1	6.708209599
	pectinesterase 2	XP_002264156.1	6.585362451
	UPF0481 protein At3g47200-like	XP_010659621.1	6.55216031

[z] Genscope accession number (NCBI *Vitis vinifera* Annotation Release 101 genome)

Appendix II: Supplementary Figures

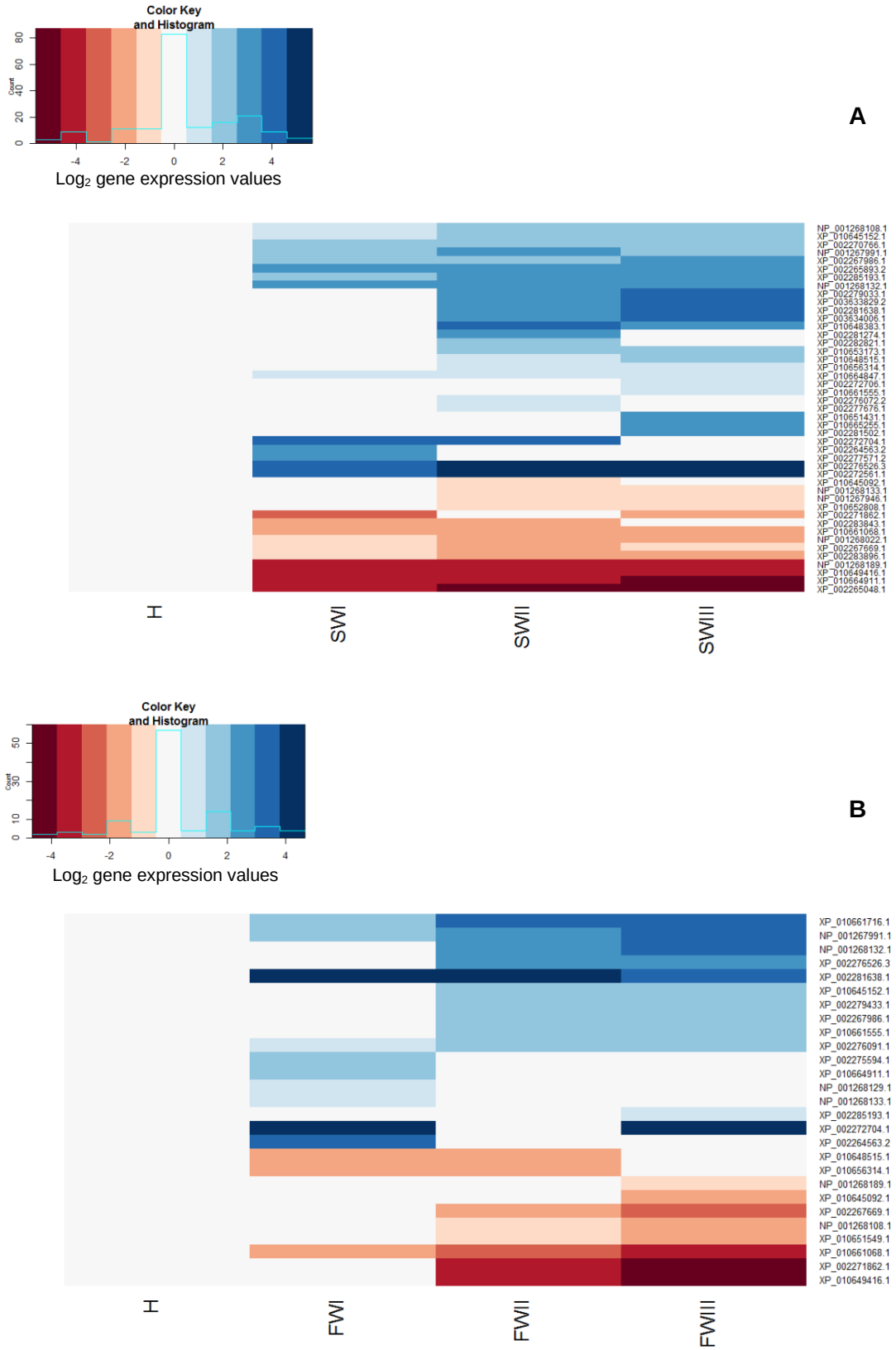


Figure S1 Heat maps demonstrating expression profiles for differentially expressed MYB genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

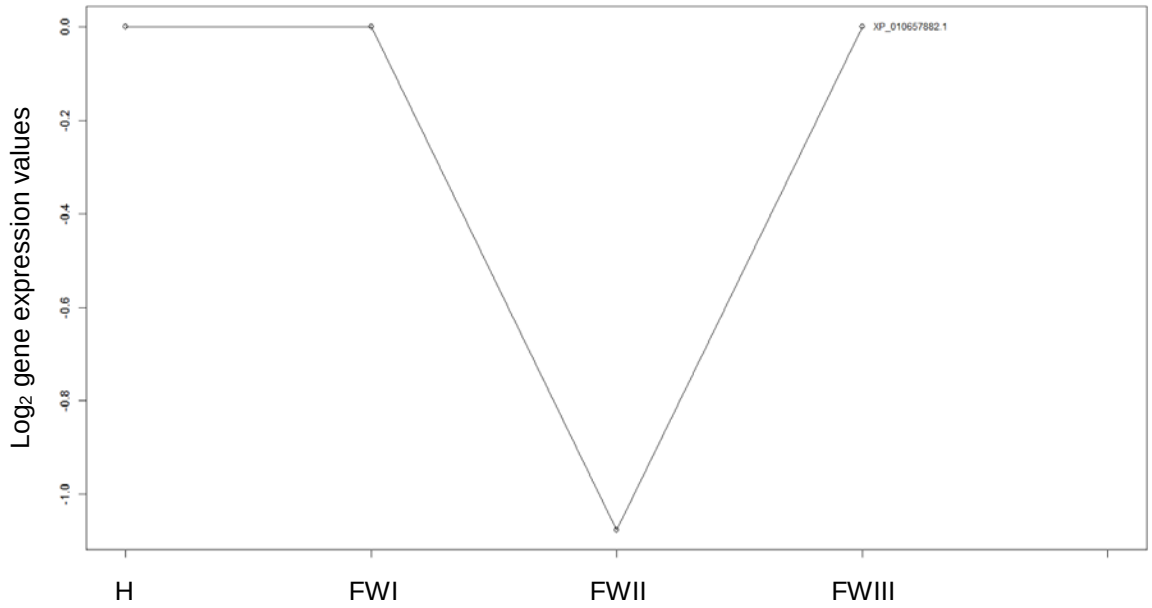


Figure S2 Line graph demonstrating the expression profile for a differentially expressed UDP-glucose:flavonoid 3-O-glucosyltransferase gene (log_2 values) over time during fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

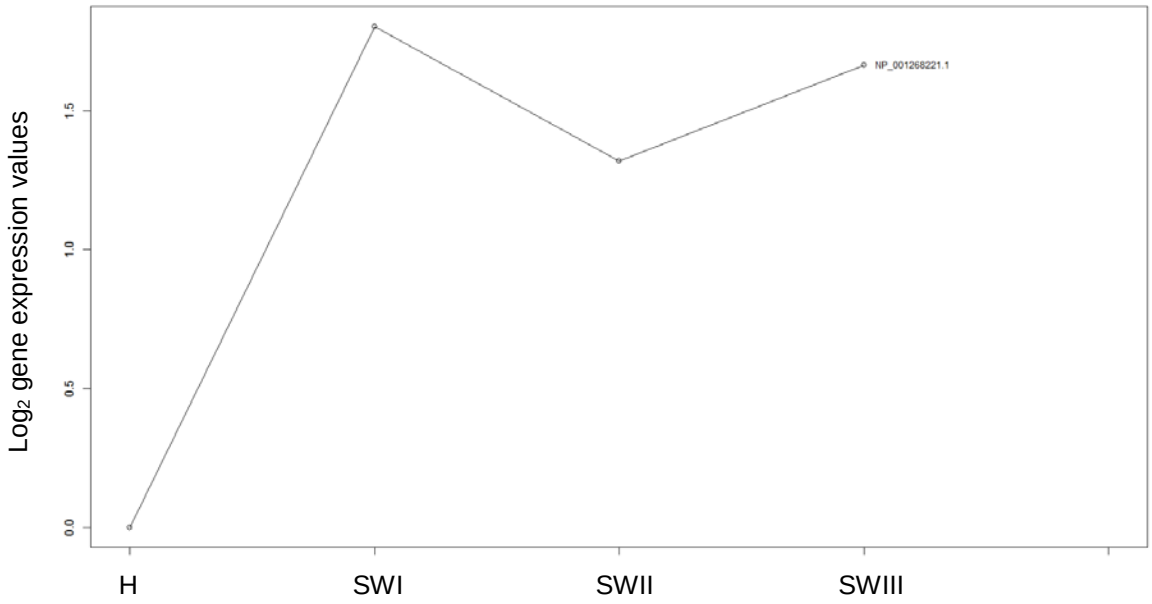


Figure S3 Line graph demonstrating the expression profile for a differentially expressed dehydrin gene (log_2 values) over time during slow postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

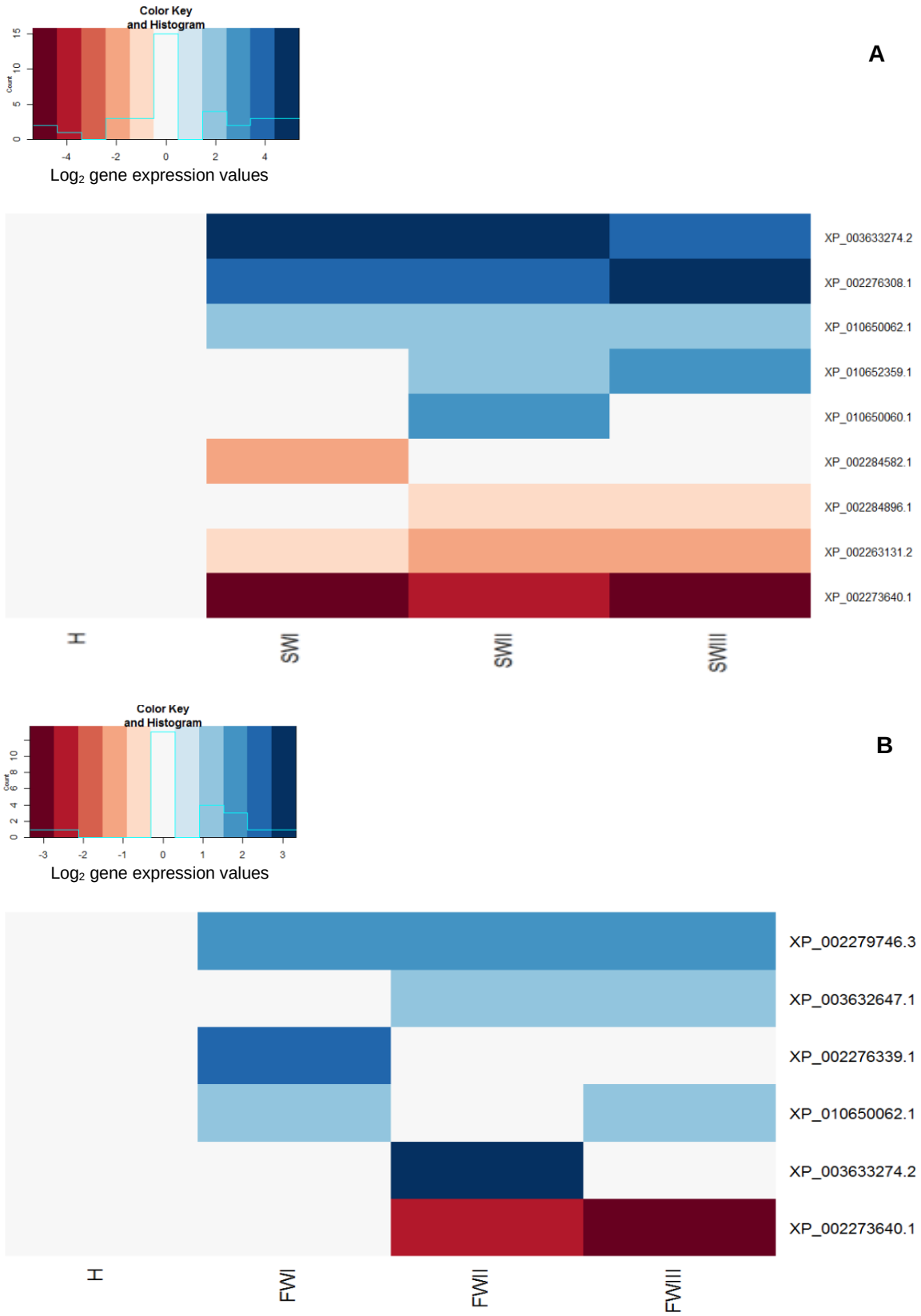


Figure S4 Heat maps demonstrating expression profiles for differentially expressed 1-aminocyclopropane-1-carboxylate oxidase genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.

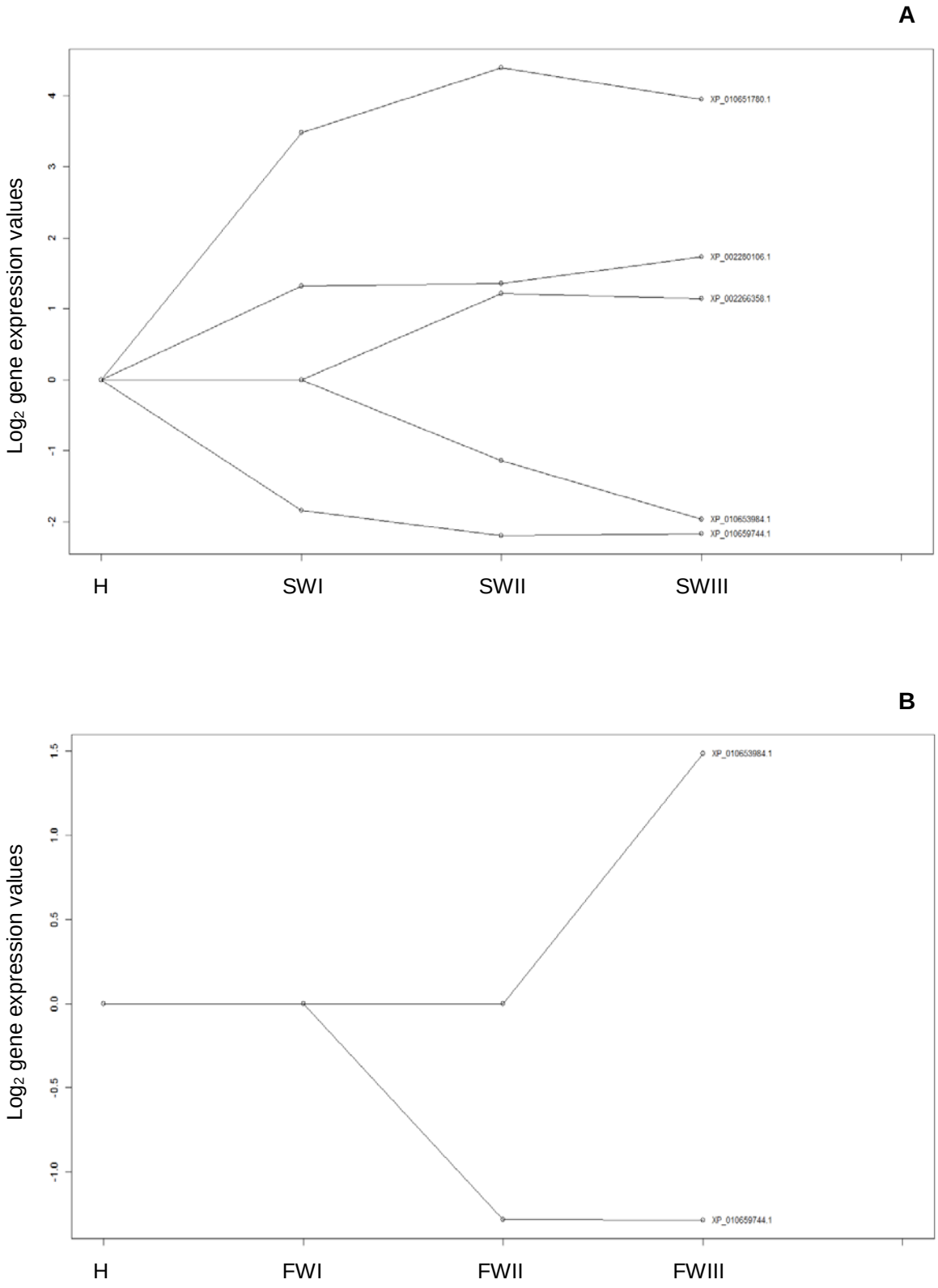


Figure S5 Line graphs demonstrating expression profiles for differentially expressed *S*-adenosyl methionine synthase genes (log_2 values) over time during (**A**) slow and (**B**) fast postharvest withering in *Vitis vinifera* 'Cabernet Franc' berries.